Knockdown surface expression of p75 neurotrophin receptor with ER retained intrabody in mammalian cells

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Abbreviations

°C	Degree Celsius
Ab	Antibody
Aβ	Amyloid β
Abs	Antibodies
AD	Alzheimer's disease
Akt	Protein kinase B
AP	Alkaline phosphatase
APC	Allophycocyanin
APS	Ammonium persulphate
ATCC	American type culture collection
BDNF	brain-derived neurotrophic factor
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDRs	Complementarity-determining regions
CH	Constant domain of heavy chain
CIP	Calf intestinal alkaline phosphatase
CL	Constant domain of light chain
CNS	Central nervous system
CRDs	cysteine-rich domains
Cy	Cyanine Dye
Da	Dalton
ddH_2O	Double distilled water
DIV	Days *in vitro*
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
dsDNA	Double-stranded DNA
E. coli	*Escherichia coli*
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	Ethylenediaminetetraacetic acid
EGFP-F	Farnesylated enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum

ERAD	ER-associated protein degradation
ex	Extracellular domain
Fab	Fragment antigen binding region
FACS	Fluorescence Assisted Cell Sorting
FAIM	fas apoptosis inhibitor molecule
Fc	Fragment crystallizable region
FCS	Fetal calf serum
Fig.	Figure
FR	Framework regions
FSC	Forward scatter
x g	Gravitational acceleration
G-418	Geneticin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEFs	Guanine exchange factors
GPI	Glycosylphosphatidylinositol
GRP78/BiP	Glucose-regulated protein 78
GRP94	ER resident 94 kDa glucose-regulated protein
HC	Heavy chain
HEK	Human embryonic kidney
His	Histidine
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
Ig	Immunoglobulin
Igs	Immunoglobulins
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRAK	Interleukin-1 receptor-associated kinase
IRES	Internal ribosomal entry site
JNK	Jun amino-terminal kinase
KDEL	ER-retention signal *Lys-Asp-Glu-Leu*
lac Pr.	lac Z promoter
LC	Light chain
LTD	Long-term depression
m	Meter
M	Molar
MAG	Myelin-associated glycoprotein
MAP	Ras-mitogen-activating protein
MPBST	Milk powder PBS-T solution
NF-κB	Nuclear factor kappa B
NGF	Nerve growth factor

NgR	Nogo receptor
NT-3	Neurotrophin 3
NT-4	Neurotrophin 4
NADE	NT-associated cell death executor
NBT	Nitro blue tetrazolium
NRIF	NT-receptor interacting factor
NSC	Neuroblastoma × spinal cord
O.D.	Optical density
OMgp	Oligodendrocyte myelin glycoprotein
P/S	Penicillin/streptomycin
P75NTR	P75 neurotrophin receptor
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PMSF	Phenylmethylsulphonyl fluoride
PVDF	Polyvinylidene fluoride
Rho-GDI	Rho guanine dissociation inhibitor
RhoA	Ras homologue member A
RIP2	Receptor-interacting protein-2
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	Rounds per minute
RPMI	Roswell park memorial institute
ROCK	Rho-activated kinase
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
scFv	Single chain fragment variable
SPR	Surface plasmon resonance
SSC	Side scatter
ssDNA	Single-stranded DNA
TAE	Tris-acetate buffer with EDTA
TEMED	Tetramethylethylenediamine
TGN	Trans-Golgi network
TMB	3,3’,5,5’-tetramethylbenzidine
TNFR	Tumor necrosis factor receptor
TRAFs	TNF receptor-associated factors
Trk	Tyrosine kinase receptors
Tris	Tris(hydroxymethyl)aminomethane

UPR	Unfolded protein response
V	Volt
v/v	Percent volume per volume
VH	Variable domain of heavy chain
VL	Variable domain of light chain
w/v	Percent weight per volume

1 Abstract

Although p75 neurotrophin receptor (p75NTR) is the first neurotrophin receptor isolated, its precise functions in physiology and underlying signaling have remained elusive for many years. In this study, endoplasmic reticulum retained intrabody (ER-intrabody) technology was applied to knockdown p75NTR surface expression in mammalian cells. By fusing with the C-terminal ER retention signal KDEL, ER-intrabody impedes target receptor protein from secretory trafficking.

Monoclonal recombinant scFvs against p75NTR were isolated by phage display. These scFvs bound to p75NTR with nanomolar affinities and were used to generate p75NTR-specific ER-intrabodies. In neuron-like cell lines PC12 and NSC19, p75NTR surface expression was significantly suppressed by the p75NTR-specific ER-intrabody construct SH325-G7-KDEL. The effect of this ER-intrabody on p75NTR surface knockdown could be maintained over a period of more than eight days without obviously activating unfolded protein response (UPR). Finally, the downregulation of p75NTR surface expression was determined in mouse hippocampal primary cultures using the ER-intrabody SH325-G7-KDEL. Sholl analysis showed that dendritic complexity of neurons was significantly increased if the p75NTR expressions were reduced on their surfaces by the ER-intrabody.

In conclusion, the novel ER-intrabody SH325-G7-KDEL inhibits the surface translocation of p75NTR and ER-intrabody knockdown technology may become a powerful tool to investigate molecular mechanisms of target neuronal receptors.

Zusammenfassung

Obwohl der Neurotrophinrezeptor p75 (p75NTR) als einer der ersten Neurotrophinrezeptoren isoliert wurde, ist seine physiologische Funktion und seine Beteiligung an Signaltransduktionswegen bis heute noch nicht vollständig erforscht. In der vorliegenden Arbeit wurden intrazellulären Antikörper (*intrabodies*) eingesetzt, um einen phänotypischen *knockdown* des Oberflächenproteins p75NTR auf Säugerzellen zu erreichen. Bei dieser Technik, die auf posttranslationaler Ebene wirkt, wird die Sekretion des Zielantigens verhindert, indem man an einen spezifischen Antikörper ein ER-Retentionssignal anhängt. Dieses Retentionssignal ist für den Verbleib des Zielproteins im ER (Endoplasmatisches Retikulum) der Zelle verantwortlich.

Mittels der *Phage Display* Technologie wurden verschiedene scFvs gegen p75NTR isoliert. Alle scFvs wiesen nanomolare Affinität gegenüber ihrem Antigen auf und wurden im weiteren Verlauf der Arbeit in ER-*intrabodies* umkloniert. Nach einer transienten Transfektion von p75NTR-positiven Zelllinien (PC12 und NSC19) mit dem *intrabody* SH325-G7-KDEL konnte mittels Durchflusszytometrie eine signifkante Herunter Regulation des p75NTR-Oberföächenproteins gezeigt werden. Dieser Effekt konnte über einen Zeitraum von acht Tagen aufrechterhalten werden, ohne dabei dem *unfolded protein response* (UPR), einen Stressindikator der Zelle, zu aktivieren. Weiterhin wurde der Effekt des *intrabody* SH325-G7-KDEL in murinen, primären Hippocampuszellen untersucht. Nach transienter Transfektion konnte mittels Sholl-Analyse gezeigt werden, dass eine reduzierte p75NTR-Oberflächenexpression zu einer signifikant erhöhten Komplexität der Neuronen führte.

Zusammenfassend wurde mit den in dieser Arbeit isolierten funktionellen ER-*intrabodies* ein weiteres Beispiel dafür gegeben, dass der posttranslationale Knockdown von Proteinen eine viel versprechende Methode ist, um die molekularen Mechanismen von Oberflächenproteinen zu untersuchen.

2 Introduction

2.1 Recombinant antibody and phage display technology

2.1.1 Antibodies and recombinant antibody fragments

Antibodies (Abs), also known as immunoglobulins (Igs), are important in the immune system to identify and neutralize exogenous intruders including toxins, bacteria, viruses, and fungi. They are produced by B cells, and found in blood or other bodily fluids of vertebrates and compose of five isotypes: IgA, IgD, IgE, IgG, and IgM. The isotypes and functions of Igs are determined by the heavy chain types. The most abundant isotype in serum for the immune system of vertebrates is IgG, which is assembled by two identical γ heavy chains (HC) and two identical κ or λ light chains (LC) connected by disulfide bonds and non-covalent bonds. The molecular masses of HC and LC are about 50 kDa and 25 kDa, respectively. Both HC and LC of IgG contain variable and constant regions. The LC is composed of one variable (VL) and one constant (CL) domain, while the HC has one variable (VH) and three constant (CH1, CH2, and CH3) domains (Delves, 2006).

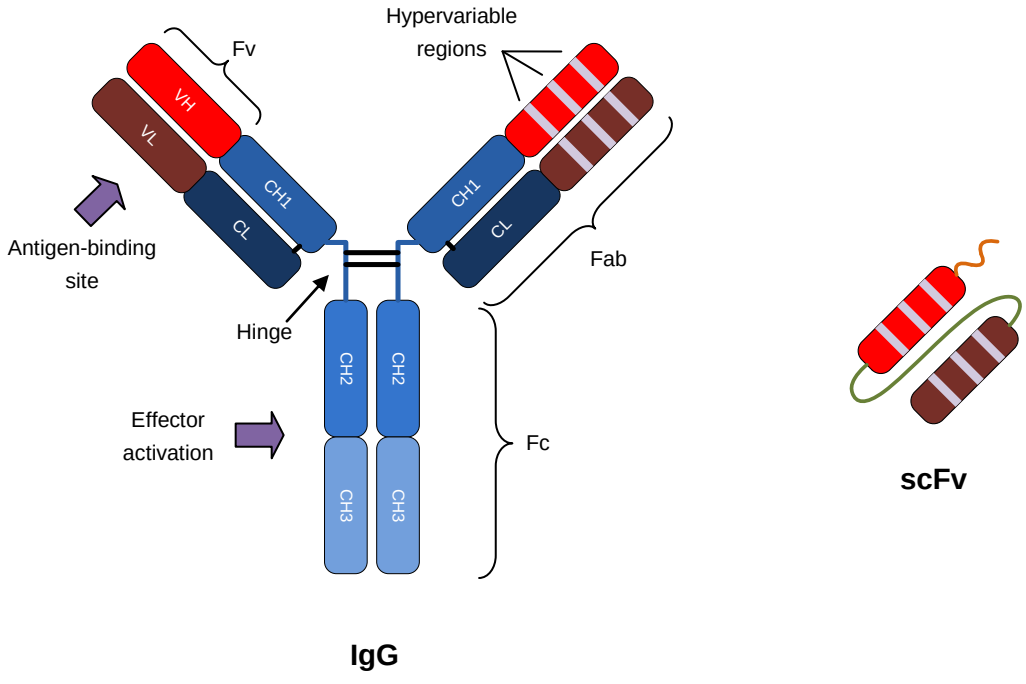

Figure 2.1 Schematic representations of IgG and recombinant scFv. Reproduced from (Dübel, 2007).

The structure of IgG, shown in Fig. 2.1, is represented as a Y shape by one Fc (fragment, crystallizable) at the bottom and two identical Fabs (fragment, antigen binding) at the tip, linked by flexible hinge regions. Fc region is responsible for effector function of an IgG to ensure that an appropriate immune response after antigen binding is mediated by the antibody. Antigen-binding site is formed by hypervariable regions of VL and VH in Fv (fragment, variable). Because antigen-binding site is complementary to the structure of epitope, hypervariable regions are also called complementarity-determining regions (CDRs). The CDRs are sequences of the highest variability and embedded into less variable framework regions (FRs) (Kabat *et al.*, 1979).

According to the structure of antibodies, it is well known that the antigen determining regions of an antibody are separated from the effector function mediating regions. Therefore, it is possible to produce antibody fragment as $F(ab')_2$, Fab, or Fv (Kontermann and Dübel, 2001). Fv fragment is the smallest antigen binding fragment containing the entire antigen binding capacity and specificity of the corresponding antibody molecule, because it consists of VL and VH domains. However, Fv fragment is normally unstable since VL and VH domain are not connected by a disulfide bond. This problem was solved by engineering the single-chain Fv (scFv) fragment (Fig. 2.1). The stability of scFvs is increased by linking the VL and VH domains with a flexible 15-20 amino acid peptide linker. The successful production of recombinant antibodies in the periplasmatic space of *E. coli* allows the generation and selection of antibody fragments because the oxidizing environment is required for the formation of disulfide bonds (Better *et al.*, 1988; Skerra and Pluckthun, 1988; Pluckthun, 1990). To date, scFv has become an important tool for research, diagnostics and therapy and has been produced in various production systems including Gram-negative and Gram-positive bacteria, yeast, fungi, insect cells as well as mammalian cells (Schirrmann *et al.*, 2008).

2.1.2 Phage display technology

Many techniques have been developed to generate monoclonal antibodies or antibody fragments with predefined antigen specificities including hybridoma technology (Köhler and Milstein, 1975), phage display (Azzazy and Highsmith, 2002), bacterial surface display (Fuchs *et al.*, 1991), ribosomal display (Hanes and Pluckthun, 1997), puromycin display (Roberts and Szostak, 1997), and yeast surface display (Boder and Wittrup, 1997). Among these methods, phage display has developed into the most robust, versatile and thus widespread selection method in the past two decades. Phage display is not only used to select antibody fragments, but also to isolate other peptides and proteins with high affinity and specificity for almost any target. This technique can also be used to study protein-ligand

interactions, receptor and antibody-binding sites, and to improve or modify the affinity of proteins for their binding partners (Azzazy and Highsmith, 2002).

Filamentous bacteriophage

The groundbreaking work for phage display was achieved by G. P. Smith based on filamentous bacteriophage M13 (Smith, 1985). Bacteriophages, or simply phages, are viruses that infect a variety of Gram-negative bacteria, such as *E. coli*, which produce and secrete phage particles without undergoing lysis. The best characterized filamentous phages, M13, f1 and fd, belong to the Ff filamentous phage family and infect *E. coli* via their F pili. These particles are rods about 6.5 nm in diameter and 900 nm in length (Russel *et al.*, 1997). M13 phage consists of a circular single-stranded DNA (ssDNA) that contains 11 genes. The entire genome is enveloped by ~2700 copies of the 50-residue major coat protein, pVIII. Besides the major coat protein, 3-5 copies for each minor coat protein located at the terminuses of M13 phage particles. At one terminus, protein pVII and pIX, are necessary for efficient particle assembly, while protein pVI and pIII, which response to phage stability and infectivity, are expressed at the other terminus (Fig. 2.2).

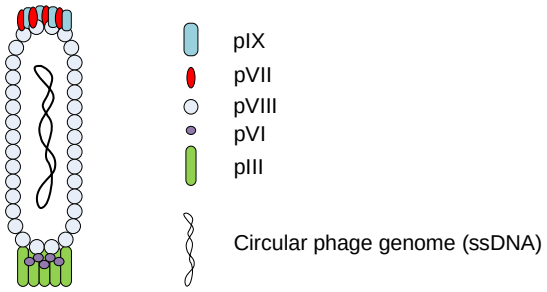

Figure 2.2 Schematic structure of M13 phage.

Phagemid and helperphage

The remarkable aspect of phage display is the linkage between the phenotype and genotype of antibody fragments. This is achieved by fusing the gene encoding antibodies typically to the minor coat pIII protein gene of M13 phage. The resulting surface presentation of this antibody::pIII fusion protein allows the selection and affinity purification of the desired gene from libraries of billions of phage clones (McCafferty *et al.*, 1990). Only the antibody fragments, Fab, Fv, scFv, and more recently scFab are used for antibody phage display, due to the limitations of *E. coli* folding machinery that complete IgG molecules can hardly be expressed in *E. coli* and displayed on the surface of phage (Better *et al.*, 1988; Skerra and

Pluckthun, 1988; Hust *et al.*, 2007a). Antibody genes can be directly fused to the wild-type pIII gene in phage genome (McCafferty *et al.*, 1990), or antibody::pIII fusion proteins can be expressed in phagemids independent from the phage genome (Hust and Dübel, 2005). Phagemid contains origins of replications of both M13 phage and *E. coli* in addition to a resistance marker and can be grown as plasmids (Mead and Kemper, 1988). Phagemid can be packaged into a complete phage with the aid of helperphage, such as M13KO7 or VCSM13, that complement all the phage genes not encoded on phagemid (Vieira and Messing, 1987). However, only 1-10% of phages display a antibody fragment by using M13KO7 for packing, due to the competition of antibody::pIII fusion protein with wildtype pIII during phage assembly (Rondot *et al.*, 2001). In 2001, Rondot et al. induced a novel helperphage, namely hyperphage, resulting in a significant increase in the fraction of phages displaying antibody fragments. Hyperphage has a truncated pIII gene on the phage genome; thereby the antibody::pIII fusion protein encoded on the phagemid is the only source for complete phage assembly. A number of phagemids have been constructed such as pHAL14 shown in Fig. 2.3 (Hust *et al.*, 2007b).

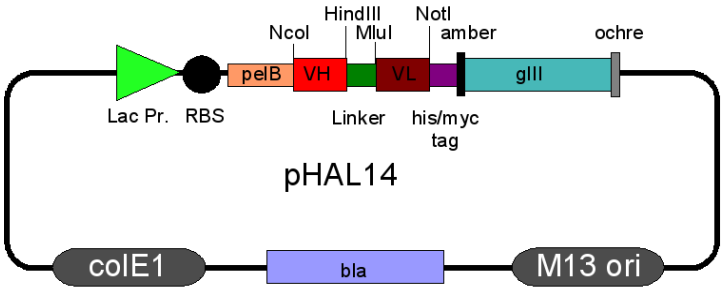

Figure 2.3 Schematic representation of pHAL14 phagemid. Lac Pr.: Lac Z promoter; RBS: ribosomal binding site; pelB: pelB leader from the pectate lyase gene of *Erwinia caratovora*; VH: variable heavy chain; VL: viarable light chain; gIII: gene III encoding pIII protein; colE1: colE1 replication origin for phagemid; bla: beta-lactamase; M13 ori: replication origin for M13 phage. The drawing was originally designed by Dr. Michael Hust (Institute for biochemistry and biotechnology, TU Braunschweig).

The pHAL14 vector is derived from the phagemid vector pHAL1 and expresses a scFv cloning cassette by cloning VH into *Nco*I/*Hind*III site and VL into *Mlu*I/*Not*I site. It contains a lac Z promoter (lac Pr.) derived from the lactose operon (Jacob and Monod, 1961) and a YoI linker connecting VH and VL for library cloning (Breitling *et al.*, 1991). In order to target the antibody fragments to the periplasmatic space of *E. coli*, a pelB leader from the pectate lyase gene of *Erwinia caratovora* is ligated to the 5' end of the antibody::pIII encoding sequence (Skerra and Pluckthun, 1988). The antibody expressed from the pHAL14 vector includes a His_6 tag and a c-myc tag for detection and purification.

Antibody library

The genetic diversity of an antibody library is essential for selecting specific antibodies efficiently. There are four types of antibody library that have been constructed. The amplification of variable genes of IgG secreting plasma cells from immunized donors is used to generate immune libraries (Clackson *et al.*, 1991). Immune libraries are normally used to generate antibodies to a specific antigen such as an infectious pathogen, therefore popular in medical research. Compared to immune libraries, naïve, semi-synthetic and synthetic libraries are known as 'single-pot' libraries according to their capacities to isolate antibodies against any possible antigen. Naïve libraries are constructed from rearranged antibody genes from Ig secreting B-cells of non-immunized donors. Semi-synthetic libraries can be created from unrearranged variable genes from pre-B cells (germline cells), or from a single antibody framework with at least one CDR region, typically CDR3 of the heavy chain for its high diversity, genetically randomized. The fully synthetic libraries are derived from a human framework with randomly integrated CDR cassettes (Hayashi *et al.*, 1994; Pini *et al.*, 1998; de Haard *et al.*, 1999). Up to date, single-pot antibody libraries with a theoretical diversity of up to 10^{11} independent clones have been generated and used for the selection of antibodies for diagnostic and therapeutic purposes (Thie *et al.*, 2008).

Panning

The technique for selecting specific antibodies *in vitro* via screening and enriching the antibody libraries are known as panning (Parmley and Smith, 1988). A typical selection cycle is illustrated in Fig. 2.4. The antigen of interest is immobilized on a solid surface, for instance 96-well polystyrole microtiter plates (Barbas *et al.*, 1991), nitrocellulose (Hawlisch *et al.*, 2001), polystyrole tubes (Hust *et al.*, 2002), or magnetic beads (Moghaddam *et al.*, 2003). The microtiter plates and polystyrole tubes are most widely used. The conformational integrity of antigens during the immobilization is critical to obtain functionally specific antibodies. Some antibodies selected against an adsorbed antigen may not be able to recognize the native form antigen (Sanna *et al.*, 1995). Indirect antigen immobilization using antigen-specific capture antibodies may avoid this problem.

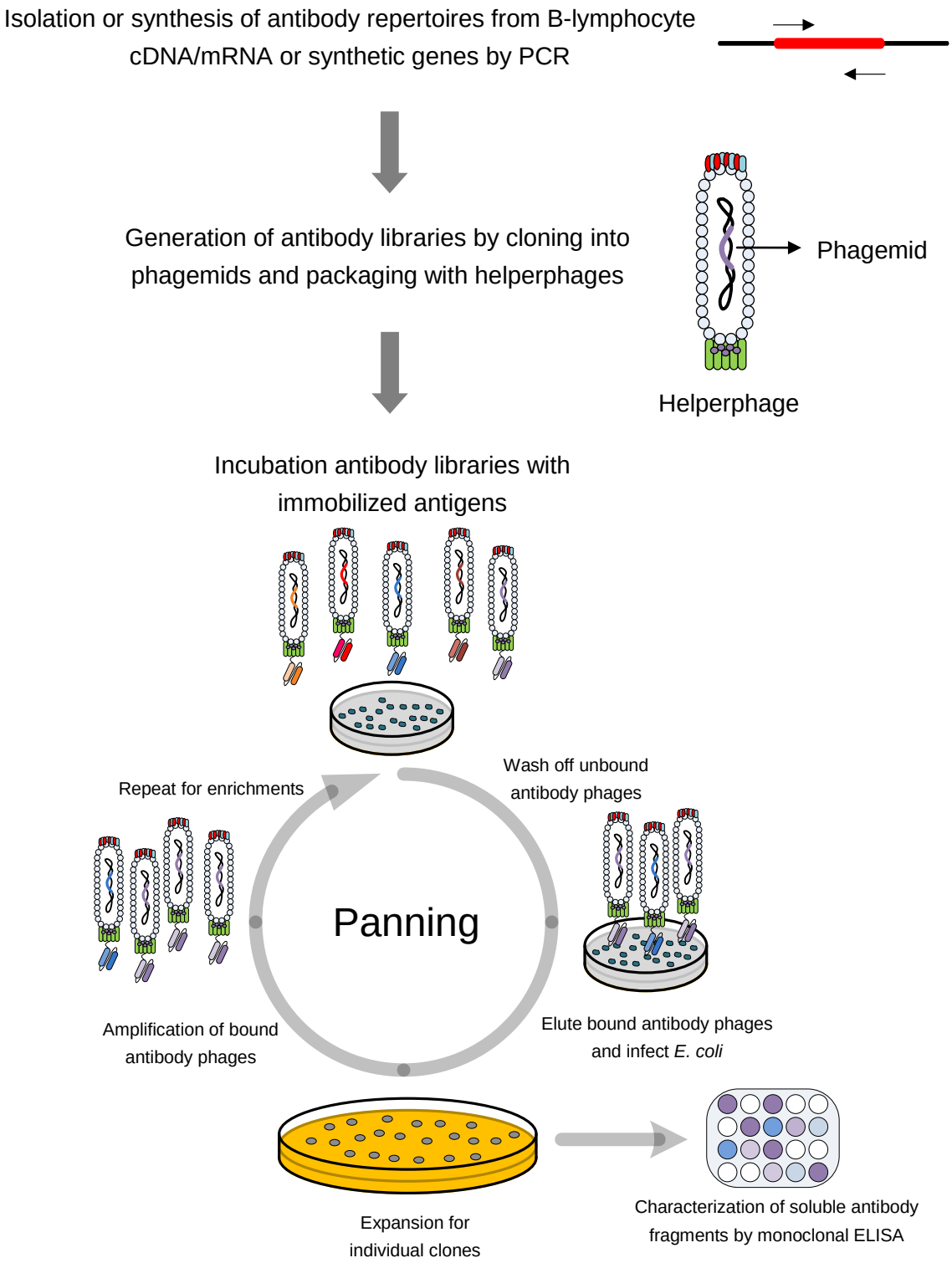

Figure 2.4 Schematic description of the selection of antibody fragments from antibody libraries by phage display (panning).

Antibody phages are firstly incubated with immobilized antigens. Unbound antibody phages are then removed by thorough washing. Bound phages are subsequently eluted by changing the binding conditions and re-amplified by infection of *E. coli*. Because the binding of nonspecific phage limits the enrichment achieved per cycle, usually 2-6 panning rounds are necessary to select specifically antibody phages in practice. In the end, individual antibody clones can be identified by monoclonal ELISA. More details about panning can be seen in Kontermann and Dübel (2001).

2.2 ER-intrabody knockdown technology

2.2.1 Intracellular antibody

Intracellular antibodies, or called intrabodies, are designed to be expressed and function within intracellular compartments. Due to the development of recombinant antibody engineering, various antibody formats have been used as intrabodies including Fabs, scFvs, single-chain diabodies, or even intact IgG molecules (Kontermann, 2004). Because of the small size, scFvs are easily expressed and assembled as functional molecules intracellularly, therefore the most commonly formats applied. In the past decade, more and more intrabodies have been isolated from phage display libraries and used in a wide range of applications, including HIV infection, tumor therapy, tissue transplantation, and treatments of neurological disorders (Kontermann, 2004; Miller and Messer, 2005; Boldicke, 2007).

According to high specificities and high affinities to target antigens, intrabodies have been used as a biotechnological tool to block intermolecular interactions, and to modulate proper functions of defined target proteins at the posttranslational level. In order to exert their functions, intrabodies have to be directed to and/or retained in various subcellular locations including the cytosol, nucleus, endoplasmic reticulum (ER), mitochondria, peroxisome, plasma membrane and trans-Golgi network (TGN) through fusion to a variety of signal and/or retention peptides. For instance, an intrabody can be trafficked to ER by an antibody signal peptide and retarded by constructing an ER retention signal at carboxy-terminal (Boldicke, 2007). In addition, farnesylation signals or nuclear localization signals (NLS) can direct intrabodies to plasma membrane or nucleus in mammalian cells effectively (Yoneda *et al.*, 1992; Kalejta *et al.*, 1997).

2.2.2 ER-intrabody

Intrabodies combined with the ER retention peptides are known as ER retained intrabodies or ER-intrabodies (Fig. 2.5). KDEL is one of the most common ER retention sequences. It is a conserved carboxy- terminal tetrapeptide (*Lys-Asp-Glu-Leu*) that serves as a retrieval signal to retain the majority of ER resident proteins within the ER.

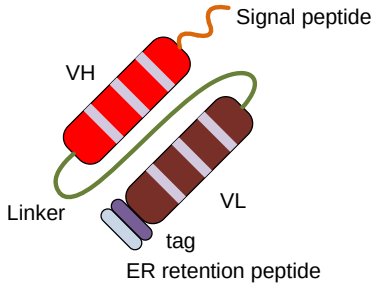

Figure 2.5 Schematic representation of an ER-intrabody in scFv format. VH: variable domain of heavy chain; VL: variable domain of light chain; Linker: a flexible 15-20 amino acid peptide linking VH and VL domains.

The KDEL signal has been demonstrated to be recognized by a receptor that is encoded by the ERD2 gene, and a human homologue of ERD2 (hERD2) has been identified (Lewis and Pelham, 1992a). Soon afterwards, Lewis and Pelham identified another related human receptor with similar function of human ERD2 (Lewis and Pelham, 1992b). After induction of hERD2 oligomerization by KDEL, COPI-coated budding protein complexes are formed containing ER resident proteins and hERD2 receptors. Associated with ADP-ribosylation factor GTPase activating protein (ArfGAP), the protein complexes are recycled back to the ER. The affinity of KDEL receptors for KDEL is pH-dependent (Wilson *et al.*, 1993). Due to the pH differences, the KDEL sequence binds to the KDEL receptor with a high affinity at the acidic pH of the Golgi lumen and releases from the KDEL receptor after retrieval to the ER because the affinity is decreased by the neutral pH of the ER lumen. The proteins dissociated from the receptors may be degraded by the cytoplasmic proteosome according to ER-associated protein degradation (ERAD) (Meusser *et al.*, 2005).

2.2.3 Mechanism of ER-intrabody knockdown technology

Combining the high specificity and affinity of intrabodies and the retention function of the KDEL peptide, ER-intrabodies inhibit the translocation of specific target surface molecules from the ER to the cell surface (Munro and Pelham, 1987; Boldicke, 2007), which results in a very efficient knockdown of the cell surface expressions of specific target molecules (Fig. 2.6).

In a mammalian cell, target surface proteins are continuously expressed and transported to the surface (Fig. 2.6A). After introducing the plasmid of ER-intrabody specific to the target (Fig. 2.6B), ER-intrabodies are expressed and bind to the target proteins within the ER to form the complexes consisting of ER-intrabodies and target proteins (Fig. 2.6C). Due to the KDEL peptide, the complexes are translocated in COPI-coated vesicles and then recycled between the Golgi complex and the ER (Fig 2.6C). Finally, the knockdown, or even phenotypic knockout of the target proteins from the cell surface is achieved (Fig. 2.6D).

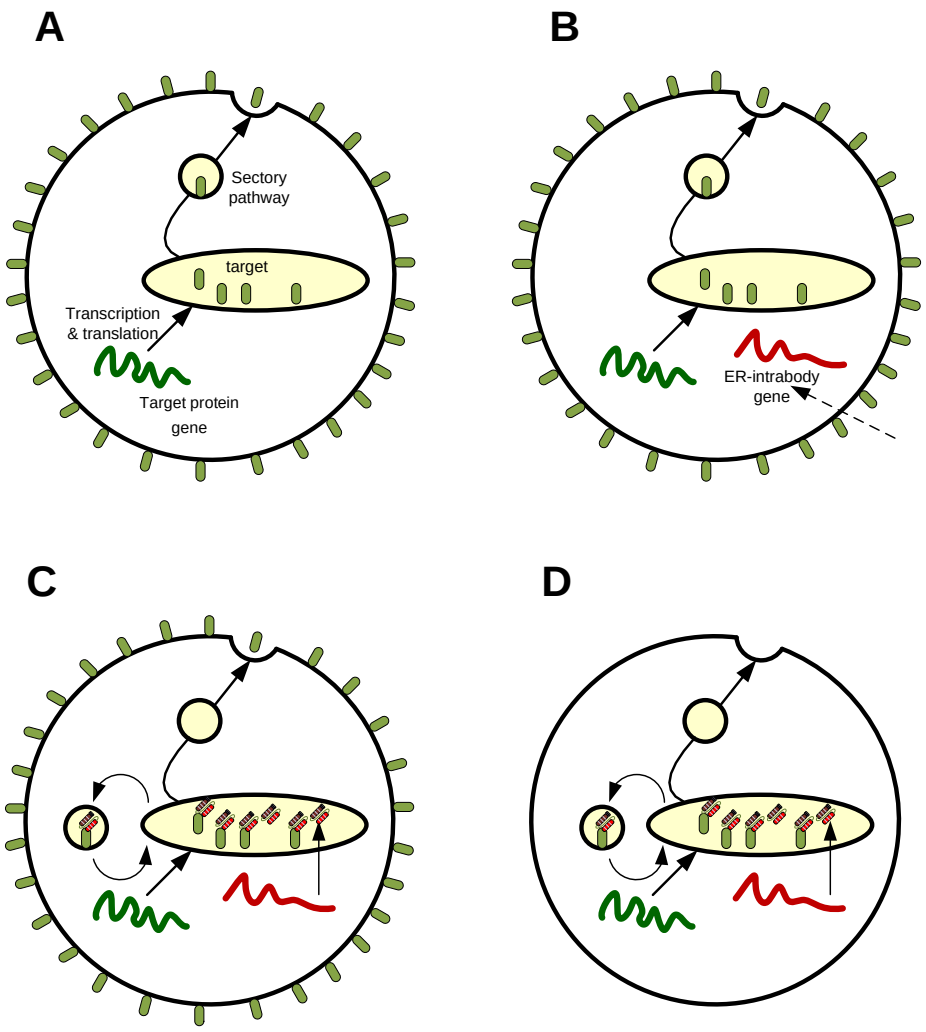

Figure 2.6 ER-intrabody in a target surface molecule knockdown. (A) The target proteins (shown in green) are expressed and transported to the cell surface. (B) The plasmids (shown in red) encoding ER-intrabody specific to the target proteins are introduced and expressed. (C) Due to the KDEL peptide, the complexes of ER-intrabodies and the target proteins are translocated in the COPI-coated vesicles and recycled between the Golgi complex and the ER. (D) The surface expression of target proteins is inhibited. (Modified based upon an unpublished drawing by S. Dübel).

ER-intrabodies are potentials to study specific protein functions and can also be used in therapeutic applications. The crosstalk between ErbB-2 and the androgen receptor was

verified by applying an anti-ErbB-2 ER-intrabody (Liu *et al.*, 2005). In spite of the limitation of gene delivery, intraperitoneal delivery of adenovirus encoding the anti-ErbB-2 ER-intrabody enhanced survival and reduces tumor growth in a xenograft model of human ovarian carcinoma in SCID mice (Deshane *et al.*, 1997). Furthermore, ER-intrabodies have been broadly used to interrupt the HIV-1 viral life cycle. It has been shown that the HIV-1 glycoprotein gp120, its precursor gp160 and corresponding co-receptors CCR5 or CXCR4 were targeted by ER-intrabodies, and the virus-mediated syncytial formation was subsequently blocked, which led to low infectivity of progeny HIV particles (Boldicke, 2007). ER-intrabodies have also been applied in neurodegenerative disorders, for example, to study the biogenesis of Alzheimer's disease (AD) and prion diseases. It has been determined that the generation of Aβ and PrPsc, which play important roles in AD and prion diseases respectively, can be experimentally inhibited by ER-intrabodies (Paganetti *et al.*, 2005; Vetrugno *et al.*, 2005).

2.3 P75 neurotrophin receptor (p75NTR)

P75 neurotrophin receptor (p75NTR) was the first neurotrophin receptor identified; however, its precise physiological role has remained elusive for many years. In contrast to the tyrosine kinase receptor (Trk) family, p75NTR has a similar low affinity without selectivity for all neurotrophins (NT), which have been known as the essential factors in the development and functioning of the nervous system (Arevalo and Wu, 2006). Four neurotrophins have been identified in mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4 (NT-4) (Lu *et al.*, 2005). Recent work also found that neurotrophins arise from precursors, known as proneurotrophins, which will bind p75NTR with high affinity (Lee *et al.*, 2001). The expression of p75NTR changes during the life span of an organism. It is abundantly expressed throughout the central nervous system (CNS) on neurons and glia during development period, while its expression decreases remarkably in adult animals. In addition, the expression of p75NTR can be induced in the neurons after injury (Chao, 2003). P75NTR is also expressed in blood vessel smooth muscle cells as well, although its role in these cells is not yet clear (von Schack *et al.*, 2001).

2.3.1 Molecular structure of p75NTR

P75NTR is a single-pass type I transmembrane protein that consists of an extracellular domain, a transmembrane domain and an intracellular domain (Fig. 2.7) (Johnson *et al.*, 1986). The structure of human p75NTR gene was identified as 75,000 base pairs in size, containing 10 exons and nine introns (Johnson *et al.*, 1986; Santee and Owen-Schaub, 1996). Full-length p75NTR includes four cysteine-rich domains (CRDs) in the extracellular

domain which are negatively charged and subsequently recognized as the defining characteristic of tumor necrosis factor receptor (TNFR) superfamily. The CRDs are encoded in exon III and the third and fourth CRDs are determined to be required for neurotrophin binding (Dechant and Barde, 2002). A stalk domain connecting the single transmembrane domain attaches to the CRDs and is involved in the sorting of p75NTR and the transmembrane domain is highly conserved across species. The intracellular part of p75NTR is composed of a juxtamembrane domain and a globular, six-helix carboxy-terminal domain, known as the death domain, which is associated in apoptosis signal transduction (Coulson *et al.*, 2000). Unlike the other members in the TNFR family, the death domain of p75NTR belongs to subtype II, which is different from the subtype I death domains of other TNFR receptors (Bender and Thorburn, 2005).

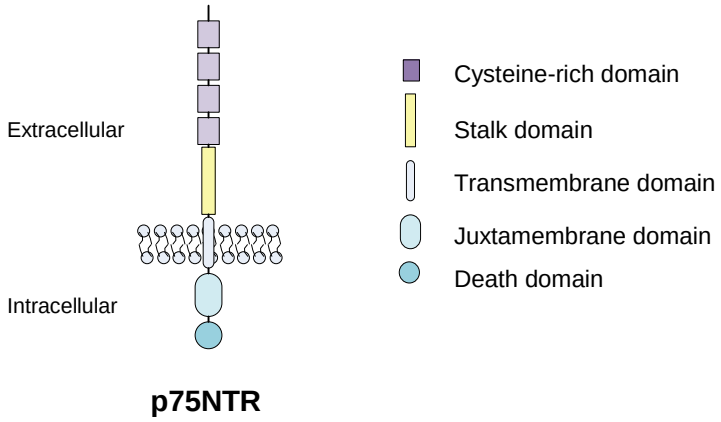

Figure 2.7 Schematic structure of p75NTR. Reproduced from Dechant and Barde (2002).

2.3.2 Diverse functions of p75NTR depending on co-receptors

P75NTR normally has to collaborate with many different protein partners to induce several cellular responses, since its intracellular region does not exhibit an intrinsic ligand-inducible enzymatic function as Trk receptors (Hempstead, 2002). Trk receptors were the first co-receptors identified for p75NTR as the p75NTR and Trk receptors interaction will increase the affinity and selectivity of neurotrophin binding (Bibel *et al.*, 1999). Besides Trk receptors, sortilin, LINGO-1, and NgR have been demonstrated as co-receptors of p75NTR to produce different biological responses (Lu *et al.*, 2005).

2.3.2.1 P75NTR regulates the cell death or cell survival in the absence of Trk receptors

P75NTR is to promote apoptotic effects in response to neurotrophins depending on the molecular and cellular context. Overexpression of p75NTR alone is enough to cause cell death (Rabizadeh *et al.*, 1993). In the absence of Trk receptors, the apoptotic effects can be mediated by p75NTR via several different pathways (Fig. 2.8), which can be counteracted by TrkA presence (Yoon *et al.*, 1998).

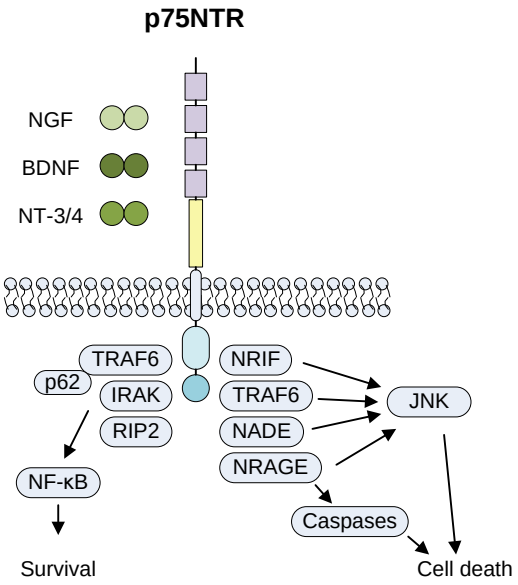

Figure 2.8 P75NTR regulates either cell death or cell survival in response to neurotrophins in the absence of Trk receptors.

Although the signaling pathways of p75NTR-dependent apoptotic response are less clear, it has been elucidated that Jun amino-terminal kinase (JNK) is involved. JNK has been determined to be activated by many interactors, including NT-receptor interacting factor (NRIF), NT-associated cell death executor (NADE), NT-receptor-interacting MAGE homolog (NRAGE) and TNF receptor-associated factors (TRAFs), which will work alone or in combination (Yeiser *et al.*, 2004). Coexpression of NRIF and TRAF6 in p75NTR-expressing cells will induce activation of JNK and promote cell death (Gentry *et al.*, 2004b). Besides JNK activation, NRAGE also interacts with p75NTR to promote apoptosis through activating caspases-3, -7, and -9 (Salehi *et al.*, 2000). The NADE-dependent signaling pathway is selectively responsible to NGF binding to p75NTR, but not BDNF or NT-3/4 binding (Mukai *et al.*, 2000).

Intriguingly, NGF binding to p75NTR leads to promote survival response by activating transcription factor, nuclear factor kappa B (NF-κB), in rat Schwann cells, although this is not

completely understood (Dechant and Barde, 1997). This activation was not observed in Schwann cells isolated from p75NTR-deficient mice. The activation of NF-κB is selective for NGF and not activated by BDNF or NT-3 (Carter *et al.*, 1996). The activation of NF-κB requires several proteins, including TRAF6, p62, interleukin-1 receptor-associated kinase (IRAK), and receptor-interacting protein-2 (RIP2) (Arevalo and Wu, 2006). Upon activation, NF-κB is translocated to the nucleus and traggers the expression of Hes 1/5 to modulate dendritic growth (Salama-Cohen *et al.*, 2005).

2.3.2.2 P75NTR acts as a pro-survival receptor in the presence of Trk receptors

P75NTR may act as a co-receptor of the Trk receptors to enhance the survival of neurons. It has been identified to augment Trk signaling by stimulating the phosphorylation of Shc, a Trk adaptor (Epa *et al.*, 2004). It has been proposed that p75NTR enhances the affinity and specificity of Trk receptors for neurotrophins. The subdomain of Trk receptor is altered by p75NTR interaction to expose a special site for neurotrophin binding (Zaccaro *et al.*, 2001). The affinity of Trk receptor to neurotrophin can increase from 10^{-9} M to 10^{-11} M. Another group found the evidence that p75NTR increased the affinity of TrkA for NGF while impeding its binding of NT-3 (Kuruvilla *et al.*, 2004). Recent research demonstrated that the crystal structure of p75NTR and NGF complex was asymmetric (He and Garcia, 2004). One p75NTR bound to a dimeric NGF and the conformation of the complex was unable to recruit another p75NTR but suggested binding to another co-receptor, such as Trk receptor, to form a heterodimeric complex. However, Wehrman and colleagues lately proposed that p75NTR and TrkA most likely communicated in the downstream signaling pathway rather than through direct extracellular interaction. They found that NGF dimerized TrkA and p75NTR existed on the cell surface as a preformed oligomer that did not dissociated by NGF (Wehrman *et al.*, 2007). Thereby it assumed that the Trk-mediated signaling was enhanced via p75NTR communicating with Trk receptors through the cytosolic and/or transmembrane domains (Nykjaer *et al.*, 2005). The signaling pathways of Trk dimer-p75NTR receptor complexes might be inherently different from those of Trk dimers. TrkA dimers are sufficient to sustain neurite outgrowth, whereas the heteromeric TrkA dimer-p75NTR complex is necessary for complete outgrowth and for long-term survival (Lad *et al.*, 2003).

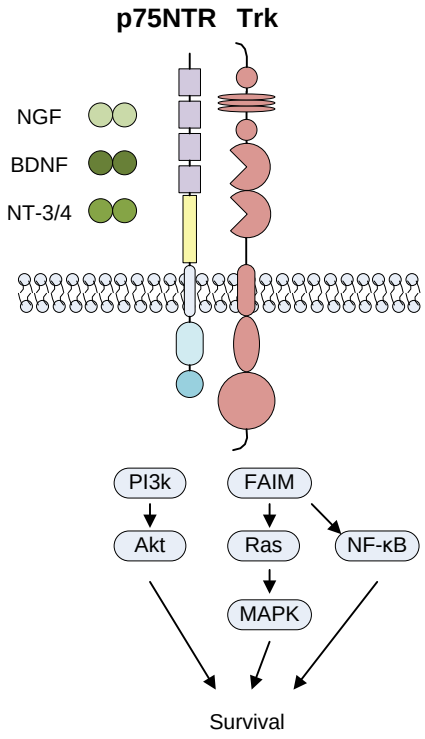

Figure 2.9 P75NTR acts as a co-receptor of Trk receptors for neurotrophins to enhance the survival of neurons.

Activated TrkA elicits the Ras-mitogen-activating protein (MAP) kinase pathway in the absence of p75NTR, but TrkA can stimulate the PI3-kinase-protein kinase B (Akt) cascade only in the presence of 75NTR (Fig. 2.9) (Nykjaer *et al.*, 2005). It has recently been identified that TrkA and p75NTR promoted NGF-dependent neurite outgrowth through activating fas apoptosis inhibitor molecule (FAIM) (Sole *et al.*, 2004). FAIM is recognized as an adaptor that can bind both TrkA and p75NTR and the neurite outgrowth is thought to invoke activation of both NF-κB and the Ras-MAP kinase pathway.

2.3.2.3 Sortilin-p75NTR complex is essential for proneurotrophin-induced neuronal cell death

P75NTR alone can activate pro-apoptotic effects via binding mature neurotrophins; however, the neurotrophin-induced p75NTR-dependent apoptosis is not efficient. High non-physiological concentrations of mature neurotrophins are often required to induce even modest levels of cell death (Barker, 2004). This raises the possibility that there are other mammalian ligands for p75NTR. Proneurotrophins, the precursors of neurotrophins, have been found binding p75NTR with high affinity and triggers cell death at much lower concentrations, compared to mature neurotrophins (Lee *et al.*, 2001). It has been

demonstrated that proNGF and proBDNF (Teng *et al.*, 2005) are the potent inducers of p75NTR-dependent apoptosis *in vitro*, such as sympathetic neurons, oligodendrocytes, and in a vascular smooth muscle cell line (Lee *et al.*, 2001; Nykjaer *et al.*, 2004) or *in vivo*, such as oligodendrocyte cells and adult corticospinal neurons (Beattie *et al.*, 2002; Harrington *et al.*, 2004). There is now evidence that proBDNF functions as an endogenous ligand for regulation of long-term depression (LTD) (Woo *et al.*, 2005). Additionally, it has been shown that proNGF does not bind TrkA and suggested that proNGF is an apoptotic ligand that is specific for p75NTR (Lee *et al.*, 2001).

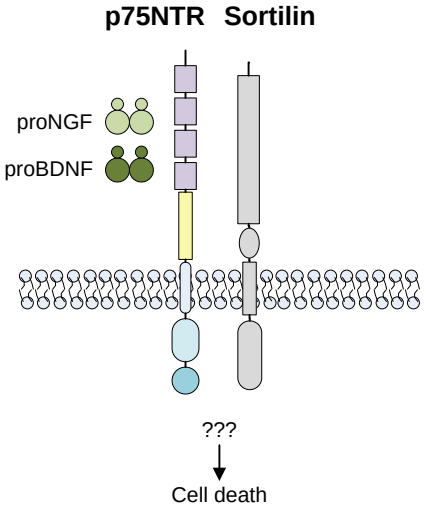

Figure 2.10 The complex consisting of p75NTR and sortilin is essential to mediate pro-apoptotic signals in response to proneurotrophins.

However, it has been implied that there should have additional membrane proteins required for the proneurotrophin-induced cell death in addition to p75NTR, since not all p75NTR-expressing cells responded to proNGF (Nykjaer *et al.*, 2004). In 2004, Nykjaer et al. reported that sortilin, a member of Vps10p-domain receptors, acted as an essential co-receptor to p75NTR for proNGF-indeced neuronal cell death (Fig. 2.10). Through crosslinking studies, they found that p75NTR and sortilin formed a receptor complex binding proNGF at the cell surface. ProNGF directly interacts with the extracellular domain of sortilin via its pro-domain, whereas it interacts with p75NTR most likely by the mature domain. The interaction between proBDNF and sortilin seems stable and proBDNF appears to be protected from proteolysis or degradation via interacting soluble sortilin (Teng *et al.*, 2005). ProBDNF is processed to 24 kDa and 13 kDa products over 4 h during incubation with plasmin, whereas proBDNF exhibits reduced degradation in the form of proBDNF–sortilin complex under comparable conditions. The signaling pathway of the p75NTR-sortilin complex has not been defined so far, but both receptors appear to be required to transduce the apoptotic effects of proNGF.

Blocking the interaction between proNGF and sortilin will inhibit proNGF-induced apoptosis, whereas introducing exogenous sortilin to Schwann cells, which normally express only p75NTR, will render these cells sensitive to the apoptotic effect of proNGF (Nykjaer et al., 2004).

2.3.2.4 NgR-Lingo-1-p75NTR complex inhibits the regeneration of neurons after injury

It is generally known that adult CNS lacks regeneration after injury. Three myelin proteins, namely myelin-associated glycoprotein (MAG), Nogo and oligodendrocyte myelin glycoprotein (OMgp), have been determined playing important roles in the inhibition of neurite outgrowth (Woolf, 2003). Nogo receptor (NgR), a glycosylphosphatidylinositol (GPI) anchor receptor, binds the myelin proteins with high affinity. However, NgR alone cannot signal any inhibition since it has no intracellular domain (Fournier *et al.*, 2001). In 2002, the NgR-p75NTR complex was confirmed by immunoprecipitation from the cells coexpressing both receptors (Wang *et al.*, 2002; Wong *et al.*, 2002). Soon after, Lingo-1 was identified to bind both NgR and p75NTR via extracellular domains (Mi *et al.*, 2004). Moreover, Mi et al. also demonstrated that the NgR-Lingo-1-p75NTR complex was essential in the signal transduction of myelin-derived inhibitors (Fig. 2.11).

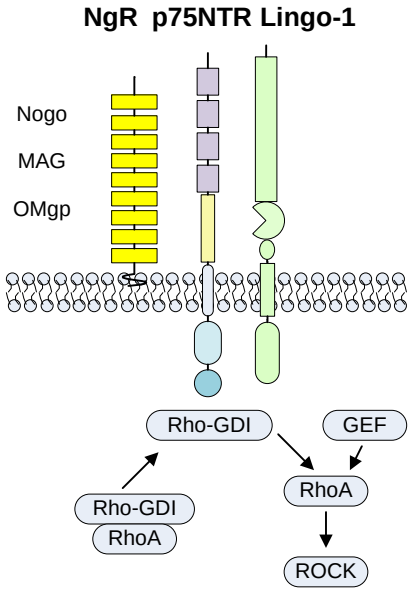

Figure 2.11 The activation of the NgR-Lingo-1-p75NTR complex by myelin proteins results in transducing an axon growth inhibition signal.

Although the precise mechanism of the NgR-Lingo-1-p75NTR complex in myelin-derived inhibition remains to be determined, it has been reported that activation of Ras homologue member A (RhoA) appears to act as a key player in inhibiting the regeneration of the CNS. Usually, RhoA is maintained in an inactive state in cells binding to Rho guanine dissociation inhibitor (Rho-GDI) (Sasaki and Takai, 1998). However, RhoA can be released from Rho-GDI as p75NTR binds to Rho-GDI with its fifth helix after myelin activates the NgR-Lingo-1-p75NTR complex. Thereby, the released RhoA can be activated by guanine exchange factors (GEFs), and activate Rho-activated kinase (ROCK), whereupon the growth cone then collapses (Yamashita and Tohyama, 2003; He and Koprivica, 2004). Nevertheless, RhoA becomes down-regulated when p75NTR binds NTs, resulting in growth cone elongation (Gehler *et al.*, 2004).

2.3.3 P75NTR in neuronal disease

Although p75NTR expression is downregulated in the adult organism, it is significantly re-expressed under certain pathological conditions of increased neuronal cell death. Emerging evidences have shown the linkage between p75NTR and many diseases including schizophrenia, cerebral edema Alzheimer's disease (AD), mechanical damage, focal ischemia, axotomy, stroke and amyotrophic lateral sclerosis (Schor, 2005). For example, amyloid β (Aβ) peptide accumulates in the brains of AD's patients and binds to p75NTR to induce p75NTR-dependent cell death (Hashimoto *et al.*, 2004). In contrast, Zhang and colleagues found that incubation of primary human neurons in culture with Aβ peptide resulted in up-regulation of p75NTR and thereby protected neurons against Aβ induced toxicity (Zhang *et al.*, 2003). The defined role of p75NTR in neuronal diseases still remains to be investigated.

2.4 Aim of this study

The goal of this study was to generate ER-intrabodies for p75NTR surface knockdown in mammalian cells. Based on phage display, scFvs specific to the extracellular domain of p75NTR were selected. The isolated scFvs were characterized by antigen binding ELISA, immunoblotting, SPR and flow cytometry. ER-intrabodies were constructed fusing the ER retention signal KDEL at the C-terminal of p75NTR-specific scFvs. Moreover, a novel bicistronic knockdown vector was generated to express the ER-intrabody and farnesylated EGFP (EGFP-F). The effects of these p75NTR-specific ER-intrabodies were examined in two distinct cell lines, PC12 and NSC19, which both express p75NTR endogenously. A time course knockdown experiment was performed in order to determine the kinetics of the p75NTR-specific ER-intrabody effect and the unfolded protein response (UPR) during

p75NTR surface downregulation process. Finally, mouse hippocampal primary cultures were applied to test p75NTR surface knockdown via the ER-intrabodies and dendrite complexities of transfected neurons were analyzed by fluorescence microscopy and Sholl analysis.

3 Materials and methods

3.1 Materials

3.1.1 Technical equipments

The technical equipments used in this study are listed in Tab. 3.1.

Table 3.1 Technical equipments

Equipment class	Type	Supplier
Blot apparatus	BioRad Trans-Blot Semi-dry SD	BioRad, Germany
Centrifuge	Eppendorf 5415D	Eppendorf AG, Germany
	Eppendorf 5810R	Eppendorf AG, Germany
	Sorvall RC6 plus	Thermo, USA
	Heraeus Biofuge pico	Heraeus, Germany
Clean bench	LaminAir HLB 2472	Heraeus, Germany
	HeraSafe	Heraeus, Germany
Cell culture incubator	Kendro Heraeus HeraCell	
	Omnilab Memmert Model 100-800	Heraeus, Germany
DNA electrophoresis	Model 40-0708	Peqlab, Germany
Electro-balance	Sartorius excellence	Sartorius, Germany
	Sartorius analytic	Sartorius, Germany
Electrophoresis apparatus	Electrophoresis power supply EPS 601/301	Amersham, Sweden
	Mini protean 3 cell	Bio-Rad, Germany
ELISA Reader	Tecan Sunrise	Tecan, Germany
ELISA washer	Columbus plus	Tecan, Germany
Flow cytometer	FC 500	Beckman Coulter, USA
	BD FACSAria™ II	BD Biosciences, USA
Microscope	Leitz Diavert	Leitz, Germany
	Axioplan 2 microscope with an Apotome®	Zeiss, Germany
Microtiter plate shaker	Microtiter plate shaker	Lab4You, Germany
PCR thermo cycler	PTC-200	MJ research, USA
pH meter	Schott Typ CG810	Schott AG, Germany
Protein purification	Äkta prime	GE Healthcare, USA
Sequencing device	ABI PRISM 310 Genetic Analyzer	A&B Applied Biosystem

Table 3.1 (continued)

Equipment class	Type	Supplier
Spectrophotometer	Nano-drop ND-1000	Thermo, USA
Surface plasmon resonance	Biacore 2000	GE Healthcare, USA
Thermo-apparatus	Thermomixer compact	Eppendorf, Germany
Trans-luminometer	iNTAS UV system	iNTAS, Germany
Vortexer	VortexGenie2	Scientific Industries, USA
Ultra-pure water device	Arium	Sartorius, Germany

3.1.2 Chemicals

All chemicals were supplied by Amersham, Invitrogen, Firmen Fluka, Merck, Riedel-de Haen, Roche Applied Science and Sigma-Aldrich. Enzymes were purchased from New England Biolabs and MBI Fermentas.

3.1.3 Buffers and solutions

The buffers and solutions used in this study are listed in Tab. 3.2. They were either prepared with double distilled water (ddH_2O) or as indicated.

Table 3.2 Buffers and solutions

Buffer and solution	Recipe
5 x Laemmli buffer	10% (w/v) SDS; 50% (v/v) Glycerin; 0.02% (w/v) Bromphenol blue; 15% (v/v) β-Mercaptoethanol
50 x TAE buffer	2 M Tris; 1 M Acetic acid; pH8.0
Ampicillin solution	100mg/mL Ampicillin
Cell lysis buffer	1% Nonidet P-40 [IGEPAL CA630 CAT NO.198596 LOT No. 9215F (ICN Biomediacals, Inc.)]; 10 mM Tris (pH 7.6); 150 mM NaCl; 5 mM EDTA; 1 mM PMSF. Protease inhibitor added before using: Aprotinin 5µg/mL; Leupeptin 5µg/mL; Pepstatin 5µg/mL
Ethidium bromide solution	10mg/mL Ethidium bromide
Gey's Balanced Salt Solution pH 7.4 (GBSS)	1.5 mM $CaCl_2 \cdot 2H_2O$; 5 mM KCl; 0.22 mM KH_2PO_4; 1 mM $MgCl_2 \cdot 6H_2O$; 0.28 $MgSO_4 \cdot 7H_2O$; 137 mM NaCl; 2.7 mM Na_2HCO_3; 0.86 mM Na_2HPO_4; 5.5 mM D-Glucose
Hank's Buffered Salt Solution (HBSS)	50 mL 10x HBSS (Invitrogen, Germany); 175 mg $NaHCO_3$; 147 mg $CaCl_2 \cdot 2H_2O$; adjusted to 500 mL with ddH_2O

Table 3.2 (continued)

Buffer and solution	Recipe
$MgCl_2$-$CaCl_2$ solution	80mM $MgCl_2$; 20mM $CaCl_2$
PBS	8 g/l NaCl, 0.2 g/l KCl; 1.44 g/l $Na_2HPO_4 \cdot 2H_2O$; 0.24 g/l KH_2PO_4; pH 7.4
PBS-T	0.05% Tween20 in PBS
PE buffer	20% (w/v) sucrose; 50 mM Tris; 1 mM EDTA; pH8
Solutions for Coomassie staining	Staining solution: 0.05% (w/v) Coomassie brilliant blue R250; 25% (v/v) Isopropanol; 10% (v/v) glycidic acetic acid Destaining solution: 10% (v/v) glycidic acetic acid
Solutions for ELISA	Coating buffer: 100 mM $NaHCO_3$, pH 9.6 FCS/PBS: 50% (v/v) FCS in PBS Developing substrate solutions: TMB solution A: 30 mM Potassium citrate; 0.5 mM Citric acid; pH 4.1 TMB solution B: 10 mM Tetramethylbenzidine (TMB); 10% (v/v) Acetone; 90% (v/v) Ethanol; 80 mM H_2O_2 Stopping solution: 1N H_2SO_4
Solutions for IMAC	10 mM imidazole buffer: 10 mM imidazole; 20 mM Na_2HPO_4; 500 mM NaCl 50 mM imidazole buffer: 50 mM imidazole; 20 mM Na_2HPO_4; 500 mM NaCl Elution buffer: 250 mM imidazole; 50 mM Na_2HPO_4; 300 mM NaCl
Solutions for SDS-PAGE	Acrylamid mix: 30% (w/v) Acrylamid; 0.8% (w/v) Bisacrylamid Ammonium persulfate (APS) solution: 10% (w/v) APS in dH_2O Tris-HCl solution: (1) 1.5M TrisHCl in dH_2O (pH 8.8) (2) 1M TrisHCl in dH_2O (pH 6.8) SDS solution: 10% (w/v) SDS in ddH_2O

Table 3.2 (continued)

Buffer and solution	Recipe
Solutions for SDS-PAGE	Running buffer: 25 mM Tris-HCl; 192 mM Glycin; 0.1% SDS
Solutions for SPR	Running PBS-T buffer: 0.005% peroxidase-free Tween20 in PBS Coupling buffer: 10mM acetic acid; 10 mM sodium acetate; pH 4.5 Regeneration buffer: 10 mM glycin buffer (pH1.5 or pH2.0)
Solutions for Western Blot	Blocking solution: 2-3% (w/v) skim milk powder in PBS Running buffer: 25mM Tris; 192mM Glycin 10x NBT solution: 70% (v/v) Dimethylformamid; 30mg NBT 10x BCIP solution: 100% (v/v) Dimethylformamid; 15mg BCIP AP Substrate buffer: 0.1M Tris; 0.5 mM $MgCl_2$; pH 9.5 50x DAB solution: 25 mg/mL DAB diluted in ddH_2O DAB substrate buffer: 0.02% cobalt (II) – chloride solution in ddH_2O

3.1.4 Medium

3.1.4.1 Prokaryotes

All media used for culturing and handling *E. coli* are shown below.

2 x YT medium	1.6% Bacto-Trypton; 1% Bacto yeast extract; 0.5% NaCl; pH7.0
2 x YT Agar	1.5% (w/v) agar in 2 x YT medium
LB medium	10 g/l Bacto-Trypton; 5 g/l Bacto-yeast extract; 10 g/l NaCl

SOC medium	2% Bacto-Trypton; 0.5% Bacto yeast extract; 0.05% NaCl; 10mM $MgCl_2$; 20mM Glucose

3.1.4.2 Eukaryotes

All reagents and media used for mammalian cell cultures are listed in Tab. 3.3 and Tab. 3.4, respectively.

Table 3.3 Reagents for mammalian cell cultures

Product	Catalog Number	Supplier
Accutase	L11-007	PAA, Austria
B27 supplement	10889038	Gibco, Invitrogen, Germany
DMEM	E15-810	PAA, Austria
DMSO	4720.1	Carl Roth, Germany
Fetal calf serum (FCS)	10084-168	Gibco, Invitrogen, USA
G418	P02-012	PAA, Austria
GlutaMAX	35050061	Gibco, Invitrogen, Germany
L-Glutamine	M11-004	PAA, Austria
Neurobasal	21103046	Gibco, Invitrogen, Germany
Penicillin/streptomycin (P/S)	P11-010	PAA, Austria
Poly-L-Lysine	P4707	Sigma, Germany
RPMI1640	E15-840	PAA, Austria
Trypsin-EDTA (1x)	L11-004	PAA, Austria

Table 3.4 Media for mammalian cell cultures

Medium	Recipe
Medium for HEK 293T cells	DMEM; 8% FCS; 1% P/S
Medium for PC12 cells	RPMI1640; 10% Horse serum; 5% FCS
Medium for NSC19 cells	DMEM, 10% FCS
Medium for dissociated hippocampus cultures	Neurobasal 50 mL; B27 supplement 2 mL; GlutaMAX (200 mM) 125 µL

3.1.5 *E. coli* strains, eukaryotic cell lines and mouse strain

All bacterial strains, eukaryotic cell lines and mouse strain are listed in Tab. 3.5 and Tab. 3.6, respectively.

Table 3.5 Bacterial strains

***E. coli* strain**	**Genotype**	**Supplier**
DH5*α*	F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *λ-thi*-1 *gyr*A96 *rel*A1 tonA	Invitrogen, Germany
XL1-Blue-MRF'	K12 strain: _(*mcr*A)183 _(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 thi-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F´ *pro*AB *lacI*qZ_M15 Tn10 (Tetr)]	Stratagene, USA

Table 3.6 Eukaryotic cell lines and mouse strain

Cell line	**Description**	**Supplier**
HEK 293T	Immortalized human embryonic kidney cells	ATCC (CRL-1573)
PC12	Immortalized rat pheochromocytoma cells	DSMZ (ACC 159)
NSC19	Neuroblastoma × spinal cord (NSC) hybrid cells (Cashman *et al.*, 1992)	Kind gift from Prof. Brigitte M. Jockusch, Zoologisches Institut, TU Braunschweig
Mouse strain	C57 Bl/6	Kind gift from Prof. Martin Korte, Institute for cellular neurobiology, TU Braunschweig

3.1.6 Plasmids

All plasmids are listed in Tab. 3.7.

Table 3.7 Plasmids

Plasmid	**Description**	**Supplier**
pCMV-hIgG1-Fc-XP	Mammalian expression vector	Modified from pCMV (Invitrogen, USA)
pCMV/myc/ER	Mammalian expression vector	Invitrogen, USA
pOPE101-XP	Prokaryotic expression vector	(Schmiedl *et al.*, 2000)
IRAVp968B1065D6	Full length mouse p75NTR	RZPD, Germany

3.1.7 Oligonucleotides

All oligonucleotides were synthesized by MWG-Biotech AG (Germany), biomers.net GmbH (Germany), or Eurofins MWG Operon (Germany). The list is shown below.

Table 3.8 Oligonucleotides

Name	Sequence (5'- 3')
CZP92	TGC CAT GGC AAA GGA GAC ATG TTC CAC AGG CAT GT
CZP93	AGA CGC GGC CGC AGG AAT GAG GTT GTC AGC GGT GCC T
MKpelB-f	GCC TAC GGC AGC CGC TGG
Mkmyc-r	GAT CCT CTT CTG AGA TGA G
CZP1	ACG CGT GCT AGC GCC CCT CTC CCT CCC CCC CCC CTA ACG
CZP2	ATG GTT GTG GCC ATG GTG TAT ACG TGT TTT TCA AAG GAA AAC CAC G
CZP8	TCG CCA CCA TGG TGA GCA AGG GCG AGG AGC TGT T
CZP9	ATC CTC TAG ATC AGG AGA GCA CAC ACT TGC AG
CZP139	ATG TGC GGC CGC AGA GGA CGG T
CZP143	GGC CGC GCG CAC TCC GAG GTG CAG CTG TTG GAG ACC GGG GGA
CZP144	GGC CGC GCG CAC TCC CAG GTG CAG CTG GTG CAG TCT GGG GGA
CZP145	GGC CGC GCG CAC TCC GAG GTG CAG CTG GTG CAG TCT GGG GGA
AMAP1	ACA GGC GCG CAC TCC CAG GTG CAG CTG GTG CAG TCT
NS20	CCA GGA GTT CAG GTG CTG

3.1.8 Antibodies

All antibodies are listed in Tab. 3.9.

Table 3.9 Antibodies

Antibody	Catalog Number	Supplier
Mouse anti-myc (9E10)	Hybridoma supernatant	AG Dübel, TU Braunschweig
mAb mouse anti-His_6	11922416001	Roche, Germany

Table 3.9 (continued)

Antibody	Catalog Number	Supplier
Goat anti-mouse IgG (Fab specific) conjugated with horseradish peroxidase (HRP)	A2304	Sigma, USA
Goat anti-mouse IgG (Fc-specific) conjugated with alkaline phosphatase (AP)	A2429	Sigma, USA
mAb mouse anti Penta-His_5 HRP conjugated	34460	Qiagen, Germany
mAb mouse anti Penta-His_5	34460	Qiagen, Germany
pAb goat anti-mouse IgG (Fc specific) HRP conjugated	A0168	Sigma, USA
mAb mouse anti-p75NTR (MLR2)	Ab61425	abcam, Cambridge, UK
Recombinant rat TrkA/Fc chimera	1056-TK	R&D systems, USA
goat anti-mouse IgG (Fc_γ specific) $F(ab')_2$ fragment allophycocyanin (APC) conjugated	115-136-071	Jackson ImmunoResarch, Germany
mAb goat anti-human IgG (Fc specific) conjugated with HRP	A0170	Sigma, USA
pAb rabbit anti-human p75NTR (intracellular)	G3231	Promega
pAb rabbit anti-human p75NTR (extracellular)	AB1554	Chemicon
Goat anti-rabbit IgG (H+L) Cy3 conjugated	111-165-003	Jackson ImmunoResearch, Germany
Rabbit anti-GAPDH	G9545	Sigma, USA

Table 3.9 (continued)

Antibody	Catalog Number	Supplier
Rabbit anti-GRP94	G4545	Sigma, USA
Goat anti-rabbit IgG (whole molecule) AP conjugated	A9919	Sigma, USA

3.1.9 Softwares

All softwares are listed in Tab. 3.10.

Table 3.10 Softwares

Software	Supplier
Cytomics CXP	Beckman Coulter, USA
EXCEL	Microsoft, USA
FinchTV 1.3.1	Geospiza, USA
ImageJ	National Institutes of Health, USA
Neurolucida and Neurolucide Explorer	Micro bright field, USA
VectorNTI	Invitrogen, USA

3.2 Methods

3.2.1 Methods of molecular biology

3.2.1.1 Polymerase chain reaction (PCR)

Phusion DNA polymerase (NEB, USA) or REDTaq polymerase (Sigma, USA) were used to amplify the DNA of interest or to identify the cloning of interest, respectively. The PCR components and programs were adjusted due to the manufacturer's instructions. All the reactions were accomplished by PTC-200 DNA engine Thermal Cycler. Examples of PCR are shown below. Annealing temperature (x) and extension time (y) are calculated for each reaction due to primers and DNA length of interest, respectively.

PCR components and program with Phusion DNA polymerase:

Components:		Program:		
5x Phusion HF Buffer	10 µL	1	98 °C	30 s
10mM dNTPs (200 µM)	1 µL	2	98 °C	10 s
Forward primer (10 pmol/µL)	2.5 µL	3	x °C	30 s
Reverse primer (10 pmol/µL)	2.5 µL	4	72 °C	y min
Template DNA (1pg~10ng)	0.2 µL	5	Go to 2	29 Cycles
Phusion DNA polymerase (2U/µL)	0.5µL	6	72 °C	10 min
Double destilled water	Adjust to 50 µL	7	16 °C	forever
		8	END	

PCR components and program with REDTaq polymerase:

Components:		Program:		
10x REDTaq Polymerase Buffer	1 µL	1	94 °C	1 min
10mM dNTPs	200 µM	2	94 °C	1 min
Forward primer (10 pmol/µL)	0.2 µM	3	x °C	45 s
Reverse primer (10 pmol/µL)	0.2 µM	4	72 °C	y min
Template DNA	One bacterial colony picked	5	Go to 2	29 Cycles
REDTaq DNA Polymerase (10 U/µL)	0.3 U	6	72 °C	10 min
Double destilled water	Adjust to 10 µL	7	16 °C	forever
		8	END	

3.2.1.2 Agarose gel electrophoresis

0.8-1.2% (w/v) agarose gels were prepared in TAE buffer to separate DNA fragments. Appropriate amount of agarose was dissolved in TAE buffer and poured into an electrophoresis chamber with ethidium bromide (EB) solution in the final concentration of 0.5 µg/mL. 6x loading dye was mixed with DNA sample. The electrophoresis was performed at 10 volt/cm for 30-50 min. The agarose gel was photographed under iNTAS UV system.

3.2.1.3 Purification of DNA fragments

DNA fragments were purified by NucleoSpin Extract II kit (Macherey & Nagel, Germany) after PCR, enzymatic restriction or agarose gel electrophoresis. DNA was eluted with ddH_2O.

3.2.1.4 Enzymatic restriction of DNA

DNA samples were digested by restriction endonucleases supplied by New England Biolabs (NEB, USA) or MBI Fermentas (Germany). Reaction parameters such as buffer, temperature and incubation time were varied according to the manufacturer's instructions. Restriction enzyme was added at the last step.

3.2.1.5 Dephosphorylation of DNA fragment

Removal of the 5'-terminal phosphates from the double-stranded DNA (dsDNA) ends prevents vector from self-ligation. 1 µL of Calf Intestinal Alkaline Phosphatase (CIP, NEB, USA) was used to dephosphorate about 100 ng of purified DNA fragment with recommend buffer. The reaction was performed at 37 °C for 30 min.

3.2.1.6 Ligation of DNA

DNA fragments were ligated using T4 DNA ligase (Promega, Germany) according to the manufacturer's instruction. The whole reaction system was adjusted to 10µL with ddH_2O. The reaction was performed at 16 °C overnight.

3.2.1.7 Plasmid construction

Standard cloning procedures were performed as described (Sambrook *et al.*, 1989).

Expression vector for antigen production in human embryonic kidney (HEK) 293T cells:

A mouse p75NTRex-Fc fusion construct was generated by cloning the mouse p75NTR extracellular domain via *Nco*I and *Not*I into a modified pCMV vector containing the human IgG1 Fc domain (Fig. 3.1).

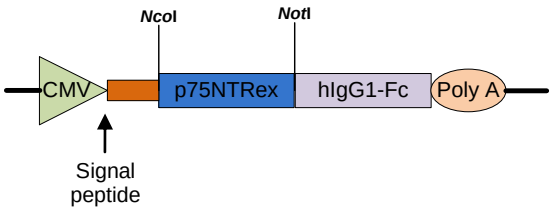

Figure 3.1 Schematic representation of the mammalian expression vector for producing antigen for phage display. CMV: CMV immediate early promoter; p75NTRex: the extracellular domain of p75NTR; hIgG1-Fc: human IgG1 Fc domain; PolyA: BGH polyadenylation sequence.

The cDNA of extracellular domain of mouse p75NTR was obtained from RZPD (German Science Centre for Genome Research, Germany) by PCR using primer pair of CZP92 (5'-TGC CAT GGC AAA GGA GAC ATG TTC CAC AGG CAT GT-3') and CZP93 (5'-AGA CGC GGC CGC AGG AAT GAG GTT GTC AGC GGT GCC T-3'). The human antibody Vκ3 (hVκ3S) subfamily signal sequence and the mouse antibody heavy chain VH3 (mVH3S) subfamily signal sequence containing a synthetic intron were used for expressing the mouse p75NTRex-Fc fusion protein in HEK 293T cells. The mammalian expression vector for mouse p75NTRex-Fc fusion protein was named as pCMV-mp75NTRex-Fc.

Expression vectors for scFvs production in *E. coli*:

The p75NTR-specific scFvs SH325-A11, SH325-B6 and SH325-G7 were selected from the naïve human antibody gene library HAL4 (Kappa) and HAL7 (Lambda) (see chapter 3.2.2.4). Oligonucleotides MKpelB-f (5'-GCC TAC GGC AGC CGC TGG-3') and Mkmyc-r (5'-GAT CCT CTT CTG AGA TGA G-3') were used to obtain the scFv cDNA sequences by PCR. The *E. coli* expression vectors pOPE101-SH325-A11, pOPE101-SH325-B6, and pOPE101-SH325-G7 were generated by cloning the scFv cDNA sequences into *E. coli* expression vector pOPE101-XP (Hust *et al.*, 2007a)via *Nco*I/*Not*I.

Knockdown vectors:

The bicistronic knockdown vector encodes the p75NTR-specific ER-intrabody and a farnesylated EGFP (EGFP-F) as a reporter gene from a single transcript (Fig. 3.1).

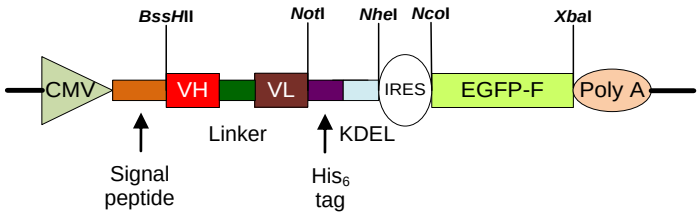

Figure 3.2 Schematic representation of the bicistronic knockdown vector. CMV: CMV immediate early promoter; VH: variable domain of heavy chain; VL: variable domain of light chain; KDEL: C-terminal ER retention signal; IRES: internal ribosomal entry site; EGFP-F: farnesylated enhanced green fluorescent protein; PolyA: BGH polyadenylation sequence.

The cDNA sequences of encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES) element and EGFP-F were obtained by PCR using primer pairs of CZP1 (5'-ACG CGT GCT AGC GCC CCT CTC CCT CCC CCC CCC CTA ACG-3')/CZP2 (5'-ATG GTT GTG GCC ATG GTG TAT ACG TGT TTT TCA AAG GAA AAC CAC G-3') and CZP8 (5'-TCG CCA CCA TGG TGA GCA AGG GCG AGG AGC TGT T-3')/CZP9 (5'-ATC CTC TAG ATC AGG AGA GCA CAC ACT TGC AG-3'), and cloned into the knockdown vector via *Nhe*I/*Nco*I and *Nco*I/*Xba*I, respectively. A sequence of His_6 tag and ER retention sequence KDEL were

constructed at C-terminal of first cistron between *Not*I and *Nhe*I. Three p75NTR-specific scFvs were amplified by PCR using the same reverse primer CZP139 (5'-ATG TGC GGC CGC AGA GGA CGG T-3') and three forward primers CZP143 (5'-GGC CGC GCG CAC TCC GAG GTG CAG CTG TTG GAG ACC GGG GGA-3'), CZP144 (5'-GGC CGC GCG CAC TCC CAG GTG CAG CTG GTG CAG TCT GGG GGA-3'), CZP145 (5'-GGC CGC GCG CAC TCC GAG GTG CAG CTG GTG CAG TCT GGG GGA-3'), respectively. The resulting PCR products were digested with *BssH*II and *Not*I and ligated into the knockdown vectors. The generated expression vectors were named pKD-*α*p75NTR-SH325-A11, pKD-*α*p75NTR-SH325-B6, and pKD-*α*p75NTR-SH325-G7. A sequence of the scFv against the hapten 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (phOx) was amplified by PCR using AMAP1 (5'-ACA GGC GCG CAC TCC CAG GTG CAG CTG GTG CAG TCT-3') and NS20 (5'-CCA GGA GTT CAG GTG CTG-3'). The p75NTR-specific scFv was substituted with the amplified phOx specific scFv in the knockdown vector and the generated expression vector was named pCMV-*α*phOx-KDEL. This vector was used as a control in later knockdown experiments.

3.2.2 Microbiological methods

3.2.2.1 Glycerol stocks

For long-term storage of bacteria, liquid culture was inoculated from a single colony and incubated in LB medium containing appropriate antibiotics at 37°C with 250 rpm overnight shaking. Freshly grown bacterial suspension was adjusted to 30% (w/v) final concentration with sterile glycerol and shock frozen in liquid nitrogen. The glycerol stocks were stored at -80°C.

3.2.2.2 Preparation of chemical competent *E. coli*

E. coli DH5*α* or XL1-Blue from glycerol storage at -80 °C was inoculated into 10 mL of LB medium without any antibiotic and then incubated at 37 °C with shaking 250 rpm overnight. 1mL of this overnight pre-culture was inoculated into 200 mL of pre-warmed LB medium in a sterilized flask, and inocubated at 37 °C, 250 rpm until the O.D.600 reached 0.4-0.6. Culture was collected into sterile ice-cold 50 mL polypropylene tubes. After cooled on ice for 10-15 min, the culture was centrifuged at 2700 x g for 5 min at 4 °C and the supernatant was carefully decanted. Pellet was resuspended by swirling or gentle vortexing in 30 mL ice-cold $MgCl_2$-$CaCl_2$ solution (80 mM $MgCl_2$, 20 mM $CaCl_2$). Subsequently, the mixture was centrifuged at 2700 x g, 4 °C for 5 min and decanted the supernatant carefully. The pellet was resuspended by swirling or gentle vortexing in 2 mL ice-cold 0.1 M $CaCl_2$ and glycerin

was added in a final dilution of 15%. These competent *E. coli* were divided into aliquots (120µL), shock frozen using liquid nitrogen, and stored at -80°C.

3.2.2.3 Transformation of *E. coli*

Chemical competent *E. coli* (~60 µL) was placed on ice for 10 min for thawing. 5 µL of ligation complex was mixed with the pre-cold competent *E. coli* and incubated on ice for 30 min. The mixture was transferred to 42 °C for 90 s and then placed on ice for 3 min for recovery. 500 µL of pre-warmed SOC medium was added into the mixture and incubated at 37°C, 800 rpm for 50 min. 100-200 µL culture was pipetted and spread gently on an agar plate with ampicillin. The agar plate was incubated with upside down at 37°C overnight after it was naturally dried.

3.2.2.4 Selection of recombinant scFvs against p75NTR extracellular domain

ScFvs against the p75NTR extracellular domain were selected from the naïve human antibody gene library HAL4 (Kappa) and HAL7 (Lambda) (Hust *et al.*, 2007b). The library was packaged using Hyperphage (Rondot *et al.*, 2001; Soltes *et al.*, 2007; Kugler *et al.*, 2008) to get polyvalent display and mixed before panning. The panning and selection was performed as described (Hust *et al.*, 2007c). The p75NTRex-Fc fusion protein was used as antigen for the panning procedure. Human IgG1 Fc (N protein SL standard, DADE Behring, Germany) was also used for preincubation of the library and for competition during the panning procedure to omit the selection of binders specific to human IgG1 Fc domain.

3.2.2.5 Production of soluble scFvs in *E. coli*

Soluble scFvs were produced in shaking flasks using the *E. coli* strain XL1-Blue MRF' according to the procedure previously described (Dübel *et al.*, 1992). 300 mL of 2xYT medium supplemented with 100 µg/mL glucose and 100 µg/mL ampicillin was inoculated with an overnight culture and the $O.D._{600}$ was adjusted to be lower than 0.1. The inoculated culture was cultured at 37 °C and 250 rpm until $O.D._{600}$ was about 0.5. Expression induction was initiated by isopropyl-beta-D-thiogalacto-pyranoside (IPTG) adjusting to the final concentration of 50 µM. After shaking for 3 hr at 30 °C, bacteria were harvested by 5,000 rpm for 5 min. The pellets were resuspended in 1/10 culture volume ice cold PE buffer and incubated for 20 min on ice with short vortexing every 5 min. The bacteria were pelleted for 30 min at 16,000 rpm, 4 °C. The expressed scFvs were purified as described in chapter 3.2.3.2.

3.2.3 Biochemical methods

3.2.3.1 Plasmid DNA preparation from *E. coli*

For miniprep, plasmid DNA was prepared from *E. coli* using NucleoSpin Plasmid kit (Macherey & Nagel, Germany). Midipreps of plasmid DNA was performed by Sigma high performance Midi Plasmid Purification Kit (Sigma, USA) if the DNA was used for transfection of mammalian cells. Plasmid DNA concentration and purity were determined by absorption measurement with 260/280 nm in Nanodrop 2000. The DNA sequence was determined by Big Dye® Terminator v1.1 Cycle Sequencing kit or GATC Biotech AG (Germany).

3.2.3.2 Immobilized metal affinity chromatography (IMAC) purification for soluble scFvs

Immobilized metal affinity chromatography (IMAC) was used for soluble scFv purifications. Chromatography was performed using 0.5 mL Chelating Sepharose™ Fast Flow (GE Healthcare, Germany) according to the manufacturer's instruction. Supernatant (periplasmic fraction) was collected and dialysed against PBS at 4 °C overnight. The dialysed periplasmic fraction was adjusted to binding condition (10 mM imidazole, 20 mM Na_2HPO_4, 500 mM NaCl) before loading. After loading, sepharose was washed with 10 mM imidazole buffer once, 50 mM imidazole buffer twice and PBS once. The soluble scFvs were eluted by elution buffer (250 mM imidazole, 50 mM Na_2HPO_4, 300 mM NaCl) and stored at -80 °C.

3.2.3.3 Enzyme-linked immunosorbent assay (ELISA)

Antigen binding ELISA

In the antigen binding ELISA, N protein SL standard, BSA, and 5 fusion proteins containing the same hIgG1 Fc domain and different neuronal receptor domains were used as controls. A BD 96-well plate (BD, USA) was coated with 100 ng of p75NTRex-Fc fusion protein or controls in 100 µL of PBS per well at 4 °C overnight. The coated plate was blocked with 100 µL of FCS per well for 1.5 hr at 37 °C and then 3x washed with PBST. The plate was subsequently incubated with 250 ng of the p75NTR-specific scFvs (see chapter 3.2.2.4) for 1.5 hr at 37 °C. After 3x washing with PBST, these scFvs were detected by mAb anti-myc tag (9E10 hybridoma, 1:500) and goat anti-mouse IgG (Fab specific) conjugated with horseradish peroxidase (HRP) (1:5,000). Signals were developed with TMB (3,3',5,5'-tetramethylbenzidine) substrate and stopped by 100 µL of 1N sulphuric acid per well. The absorbances at 450 nm with subtracting the 620 nm reference was measured by a SUNRISE microtiter plate reader.

Competition ELISA

In the competition ELISA, BD 96-well plates were coated with 100 ng of the p75NTRex-Fc fusion protein (see chapter 3.2.4.8) for each well overnight at 4 °C and blocked with FCS for 1.5 hr at 37 °C. The p75NTR-specific scFvs with serial dilution were added. After incubated for 1.5 hr at 37 °C, the plates were 3x washed with PBST. The plates were again incubated with mAb mouse anti-p75NTR (MLR2, 1:5,000) for 1.5 hr at 37 °C and 3x washed with PBST. The scFvs and mAb mouse anti-p75NTR (MLR2) were detected by mAb mouse anti Penta-His HRP conjugated (1:5,000) and pAb goat anti-mouse IgG (Fc specific) HRP conjugated (1:5,000) in corresponding plates respectively. Non-specific bindings of the HRP conjugated detection antibodies were assessed by negative control wells consisted of the scFvs combined with the pAb goat anti-mouse IgG (Fc specific) HRP conjugated or mAb mouse anti-p75NTR (MLR2) combined with mAb mouse anti Penta-His HRP conjugated. Signals were developed and measured as described in antigen binding ELISA.

3.2.3.4 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Separation and stacking gels were prepared as the components listed in Tab. 3.11 according to the sizes of samples to be separated. The separation gel was cast between the glass plates with Bio-Rad Mini Protean and overlayed by isopropanol. After gel polymerization, the isopropanol was removed, and the stacking gel was cast into the gel chamber with an appropriate comb inserted. Samples were denatured with appropriate volume of 5 x Laemmli buffer at 95 °C for 10 min before loaded to SDS-PAGE gel. Gel electrophoresis was performed under constant 200 volt for 45 min.

Table 3.11 Components for preparation of SDS-PAGE.

	Seperation gel		Stacking gel
	10%	**12%**	**4%**
ddH_2O	1.6mL	1.3mL	1.0mL
1.5M Tris-HCl, pH8.8	1mL	1mL	
1.5M Tris-HCl, pH6.8			0.20mL
10% (w/v) SDS	40µL	40µL	15µL
Acrylamide/(30%, w/v)	1.3mL	1.6mL	0.26mL
10% (w/v) APS	40µL	40µL	15µL
TEMED	2µL	2µL	2µL
Total volume	4mL	4mL	1.5mL

3.2.3.5 Coomassie Blue staining

Proteins separated by SDS-PAGE can be detected by coomassie blue solution for 2-3 hr with gently rocking. Gels were destained in 10% acetic acid solution overnight.

3.2.3.6 Western blot

One piece of PVDF membrane was saturated with methanol and two pieces of filter papers was saturated with PAGE running buffer without SDS. The transfer sandwich was positioned by placing the first filter paper, the membrane, the gel and then the second filter paper in the order from bottom to top. The process of protein transfer was executed by Bio-Rad semi-dry cell under constant voltage of 20V for 45min.

3.2.3.7 Immunostain

Detection of the p75NTRex-Fc fusion protein production

The p75NTRex-Fc elution fractions purified by ÄktaPrime (see chapter 3.2.4.8) were separated by SDS-FAGE and blotted onto a PVDF membrane. The membrane was blocked with 2% (w/v) skim milk powder PBST at 4 °C overnight. After washing with PBST, the membrane was incubated with goat anti-human IgG (Fc specific) HRP conjugated (1:10,000)

for 1 hr at RT. After 3x washing with PBST, the membrane was developed in DAB substrate for about 10-20 min.

Detection of intracellular expressions of the p75NTR-specific scFvs

To detect the intracellular expressions of p75NTR-specific scFvs, transiently transfected PC12 cell lysates (see chapter 3.2.4.7) were prepared and blotted to a PVDF membrane. The membrane was blocked with 2% (w/v) skim milk powder PBST at 4 °C overnight. The membrane was incubated with mouse anti-His_5 (1:2,000) for 1 hr at RT and followed by 3x washing with PBST. The membrane was subsequently incubated with goat anti-mouse IgG (Fab-specific) AP conjugated (1:5,000) for 1 hr at RT. Bound antibodies were visualized by NBT/BCIP substrate for about 10-20 min.

Determination of unfolded protein response (UPR)

With intent to determine the activation of UPR, transiently transfected PC12 cells from different time points (2-8 days) were sorted by BD FACSAria™ II based on the EGFP-F fluorescence (see chapter 3.2.4.6). Cell extracts of the sorted cells were blotted onto a PVDF membrane. After blocking with 2% (w/v) skim milk powder PBST at 4 °C overnight, the membrane was incubated with rabbit anti-GRP94 (1:1,000) and rabbit anti-GAPDH (1:5,000) for 1 hr at RT, separately. After 3x washing with PBST, the membrane was subsequently incubated with goat anti-rabbit IgG AP conjugated antibody (1:5,000) for 1 hr at RT. Bound antibodies were visualized by NBT/BCIP substrate for about 10-20 min.

3.2.3.8 Surface plasmon resonance (SPR)

The binding kinetics of scFvs was measured by surface plasmon resonance using a Biacore2000 and a CM5 chip (Biacore, Uppsala, Sweden). The antigen p75NTRex-Fc and control rat TrkAex-Fc (R&D systems, USA) were separately coupled on flow cells via amine coupling according to the manufacturer's instructions. The binding experiment was performed at the flow rate of 30 µL/min using PBST (0.005% peroxidase-free Tween20) at RT. Chip regenerations were done by 10 mM glycin buffers (pH1.5 or pH2.0). The p75NTR-specific scFvs with the concentrations from 2.5 nM to 200 nM were injected with an association time of 5 min and a disassociation time of 10 min. Curve fittings were done using 1:1 binding with drifting baseline.

3.2.4 Cellular methods

3.2.4.1 Cell cultures

Human embryonic kidney (HEK) 293T cell line was cultured in Dulbecco modified Eagle medium (DMEM)/high glucose (4.5 g/L) and L-glutamine (2 mM) supplemented with 8% (v/v) fetal calf serum (FCS) and 1% (v/v) penicillin/streptomycin, and cultivated at 37 °C in a 7% CO_2 incubator.

Rat pheochromocytoma PC12 cell line was cultured in RPMI 1640/L-glutamine (2 mM) supplemented with 10% horse serum (HS) and 5% FCS.

NSC19 cells were maintained in DMEM/high glucose (4.5 g/L) and L-glutamine (2 mM) supplemented with 10% (v/v) FCS. PC12 and NSC19 cells were cultivated at 37 °C in a 5% CO_2 incubator.

Cells were passaged in every 2-3 days. Cells were shortly rinsed with PBS and incubated with trypsine-EDTA until the cells detached from the bottom of cell culture plates. The reaction was stopped by FCS-containing medium. Cells were resuspended in culture medium and seeded in 1:10 (for HEK 293T) or 1:2 (for PC12 and NSC19) dilutions. Culture dishes and plates were coated with poly-L-lysine for PC12 before use.

3.2.4.2 Freezing and thawing of cells

For long-term storage, the cells were harvested by trypsine-EDTA and resuspended in freezing medium (10% DMSO and 20% FCS in relevant culture medium) for cryopreservation in 2 mL polypropylene cryo-tubes. To allow a slow and gradual freezing, the tubes were stored at -80°C in a closed styrofoam container for one day. The tubes were consequently transferred to liquid nitrogen. Before use, frozen cells were thawed in a 37°C water bath for 1-2 min and were quickly seeded in pre-warmed appropriate medium.

3.2.4.3 Cell counting

Cells were pipetted up and down for several times after tyripsinization in order to make cells as single cell suspension. To distinguish between living and dead cell, cells were 1:1 diluted in Trypan Blue dye. Living and dead cells were counted in a hemocytometer and the total cell amount was calculated.

3.2.4.4 Transient transfection

HEK 293T cells were transiently transfected using HEKFectin (Bio-Rad, Germany), while PC12 and NSC19 cells were transiently transfected using magnetofection via NeuroMag (OzBiosciences, France) according to the supplier's instructions. Culture plates needed to be coated by poly-L-lysine before seeding PC12 cells. Tab. 3.12 lists the reagent quantities for different transfections.

Table 3.12 Reagent Quantities for different transfections.

Reagent	6-well		10 cm plate	
	DNA [µg]	Reagent [µL]	DNA [µg]	Reagent [µL]
HEKfectin			10	40
NeuroMag	4	14		

3.2.4.5 Flow cytometry

2 x 10^5 of PC12 or NSC19 cells were transferred into a FACS tube (Greiner, Germany). Cells were washed with FACS buffer (5mM EDTA, 2% FCS in PBS) and centrifuged at 300 x g for 5 min at 4 °C. The cell pellets were resuspended in 100 µL of mAb mouse anti-p75NTR (MLR2, 1:200) solution or 250 ng of the p75NTR-specific scFvs in 100 µL volume, and then incubated for 1 hr on ice. The cell pellets stained by the p75NTR-specific scFvs were washed and subsequently resuspended with mAb mouse anti-His_6 (1:100) in 100 µL volume, incubating for 30 min on ice. Afterwards, all pre-stained cells were washed and incubated in 100 µL of goat anti-mouse IgG (Fc_γ specific) $F(ab')^2$ fragment allophycocyanin (APC) conjugated (1:200) for 30 min on ice. Finally, after two washing steps cells were resuspended in 500 µL of FACS buffer and kept on ice until analysis by flow cytometry using Beckman Coulter FC500. Data were analyzed by CXP analysis software. Unspecific bindings were excluded by staining cells with secondary antibody alone.

3.2.4.6 Fluorescence assisted cell sorting (FACS)

PC12 cells were transiently transfected with the knockdown vectors (see chapter 3.2.1.7) by NeuroMag. The cells from different time points (2-8 days) were trypsinized and resuspended in FACS buffer. Based on the EGFP-F expression from the knockdown transcripts, cells were sorted by BD FACSAria™ II. Only transfected cells in single suspension were interested to be harvested with appropriate gates. An example for creating the sorting gates was shown below (Fig 3.3).

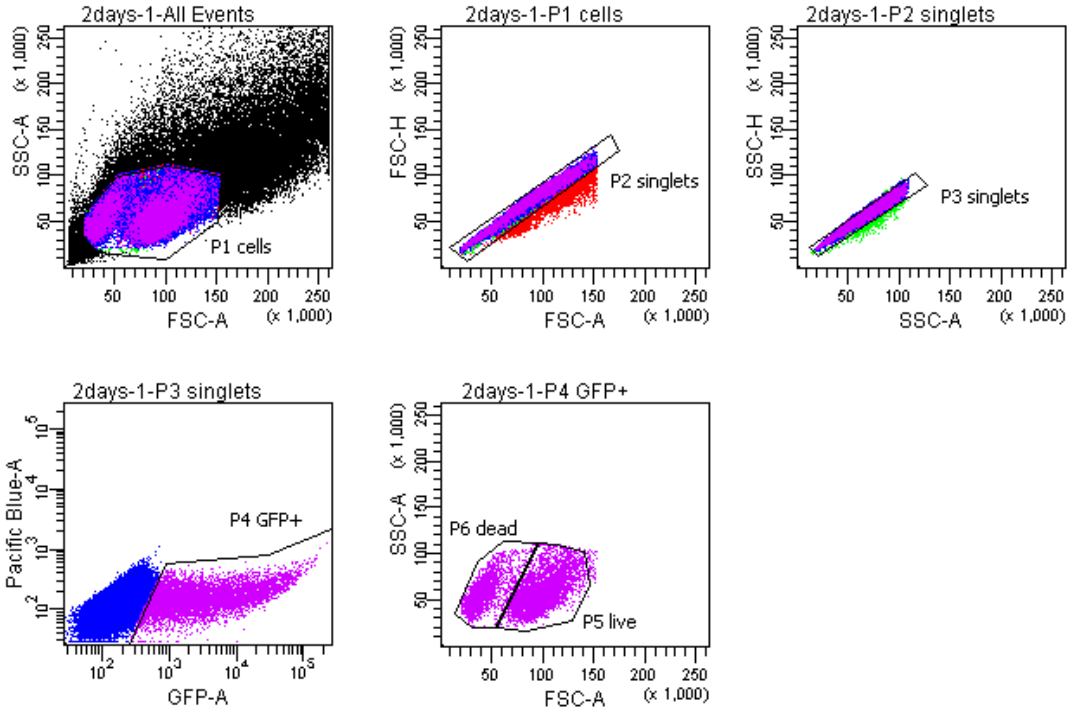

Figure 3.3 An example of cell sorting based on the EGFP fluorescence.

According to the height (H) and area (A) of forward scatter (FSC) and side scatter (SSC), gates of P1, P2, and P3 were built for the living cells in single suspension. The transfected cells were subsequently gated by P4 due to EGFP-F fluorescence. Finally, sorted cells were identified by flow cytometry, and the living and dead cells were indicated by gate P5 and P6, respectively.

3.2.4.7 Preparation of cell extracts

Cells were harvested after transient transfection. After 2x washing with pre-cold PBS, the cells were centrifuged at 1,200 rpm for 5 min. Appropriated amount of lysis buffer was used to lyse cells. After incubated on ice for 30 min, the cells were centrifuged at 4 °C, 12,000 rpm for 10 min. The supernatant was collected and stored at -20 °C until analysis.

3.2.4.8 Production of the p75NTRex-Fc fusion protein

Production of the p75NTRex-Fc fusion protein was done in HEK293T cells. 24 hr before transfection, HEK293T cells were seeded onto a 10-cm petri dish using DMEM/high glucose supplemented with 1% penicillin/streptomycin and 8% FCS. HEK293T cells were transfected by the plasmid of pCMV-mp75NTRex-Fc using HEKFectin according to the manufacturer's

recommendations. Media were changed to DMEM/high glucose supplemented with 1% penicillin/streptomycin and 4% low bovine IgG level FCS (PAA, Germany) 24 hr after transfection. Expressed p75NTRex-Fc fusion protein was collected in the supernatant every 48 hr for 14 days and purified by affinity chromatography using a HiTrap^{TM} 1 mL protein A HP column on an ÄktaPrime system according to manufacturer's instructions. Briefly, before loading the filtered samples, column was equilibrated with 20 column volumes binding buffer (20 mM sodium phosphate, pH7.0). The column was thoroughly washed with 100 mM citrate buffer (pH5.0) after sample loading. In the end, the p75NTRex-Fc fusion protein was eluted by 100 mM pH shift citrate buffer (pH2.5), followed by immediate neutralization of 1 M Tris buffer (pH9.0).

3.2.5 Methods related to mouse hippocampal primary cultures

3.2.5.1 Primary culture preparation

Primary cultures of mouse hippocampal neurons were prepared using mice (C57 Bl/6) embryonic day E18. Embryos were decapitated and the brains were kept in ice cold Gey's balanced salt solution (GBSS) supplemented with glucose. Tissue was dissociated by 30 min incubation in trypsin followed by mechanical disruption using a Pasteur pipette. Cells were plated at high density (10^5) on poly-L-lysine coated cover slips (13 mm) and kept in Neurobasal medium (Gibco) supplemented with 2% B27 (Gibco) and 0.5 mM Glutamax at 37 °C, 5% CO_2 and 99% humidity.

3.2.5.2 Transfection of primary hippocampal neurons

Primary hippocampal neurons were transfected at 7 DIV using Lipofectamine2000® (Invitrogen, Germany) according to the manufacturer's instruction. Half of the media was changed one day prior to transfection and kept at 4 °C. 0.8 µg of DNA was mixed with 2 µL of Lipofectamine in Neurobasal medium for each well. The medium was changed 50 min after transfection and replaced by half old and half new medium.

3.2.5.3 Immunocytochemistry

The immunostraining of primary hippocampal cultures was performed at RT. Four days after transfection, media were aspirated from the wells and primary hippocampal cultures (11 DIV) were incubated with pAb rabbit anti-human p75NTR (extracellular, 1:1,000) or pAb rabbit anti-human p75NTR (intracellular, 1:500) that were diluted in HBSS for 15 min. After washing with HBSS for 5 min, cultures were fixed with pre-cold 4% paraformaldehyde at 4 °C for 15

min and followed by three 20-min washing steps with PBS. The secondy antibody, goat anti-rabbit IgG Cy3 conjugated (1:500), was diluted in PBS and incubated with primary hippocampal cultures for 30 min at RT. Subsequently, two 15-min PBS washing steps were performed and the coverslips were mounted upside down on slides. After drying for 30 min at RT, culture slides were preserved at 4 °C until analysis by microscopy.

3.2.5.4 Sholl analysis

Primary hippocampal neurons were imaged using an Axioplan 2 Microscope equipped with an Apotome® controlled by the Axiovision® software. Only pyramidal neurons were considered due to their morphologies. Plain fluorescence images of hippocampal neurons were acquired by a 20x 0.8 NA Plan-APO objective (Zeiss, Germany).

Dendritic complexity of neuron was evaluated using Neurolucida® and Neurolucide Explorer software (Micro bright field, USA) for Sholl analysis (Sholl, 1953). Sholl analysis calculated the total number of dendrite crossings as an objective measurement of the total dendrite complexity. In brief, a series of concentric circles with 10 µm interval was set around the neuronal soma. Based on these circles, the number of neurites crossing each circle was counted. A statistical analysis of obtained values was carried out by Microsoft Excel using a paired student's t-test (2-tailed and 2-sample unequal variance). Significance was set at $p<0.05$.

4 Results

4.1 Production of p75NTRex-Fc fusion protein as antigen for phage display

The antigen p75NTRex-Fc was produced in order to isolate p75NTR-specific scFvs by phage display. Since p75NTR is a 75 kDa single-pass type I membrane protein (Swiss-Prot: #Q9Z0W1), its extracellular domain is firstly translocated in the ER of mammalian cells. To retain p75NTR in the cells, it is necessary to generate scFvs against the extracellular domain of p75NTR. The sequence of mouse p75NTR extracellular domain (a kind gift from RZPD, Germany) was fused to human IgG1 Fc domain to construct the p75NTRex-Fc (Fig. 3.1). HEK 293T cells were used to produce p75NTRex-Fc fusion proteins and the produced proteins were subsequently purified by affinity chromatography. The chromatogram of purification was illustrated in Fig. 4.1.

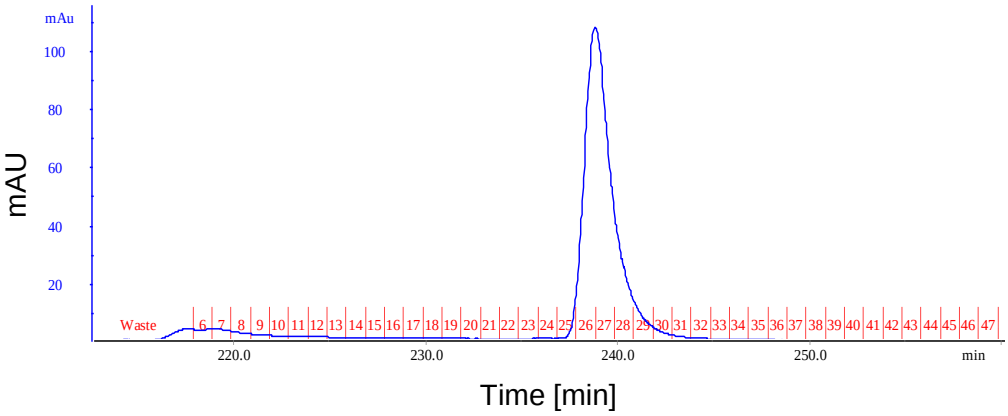

Figure 4.1 Purification of the p75NTRex-Fc fusion protein using HiTrap™ 1 mL protein A HP column. Fraction numbers are marked in read.

In order to determine the produced p75NTRex-Fc fusion protein, samples from nine elution fractions (F24-32) and one washing fraction (F6) were analyzed by immunoblotting (Fig. 4.2). Due to the glycosylation of p75NTR (Gong *et al.*, 2008) and IgG1 Fc domain (Lund *et al.*, 1996; Jefferis *et al.*, 1998), p75NTRex-Fc fusion protein migrated to ca. 100 kDa on the membrane. Concentrations of elution fractions were determined by human capture ELISA (data not shown).

Figure 4.2 Immunoblot of the purified fractions (F6, F24-32) of p75NTRex-Fc fusion protein. The p75NTRex-Fc elution fractions were obtained from ÄktaPrime purification. The membrane was incubated with goat anti-human IgG (Fc specific) HRP conjugated (1:10,000) for 1 hr at RT and developed in DAB substrate. M stands for protein marker.

4.2 Selection of recombinant scFvs against the extracellular domain of p75NTR by phage display

Phage display technology was applied to isolate antibody fragments against the extracellular domain of p75NTR. The purified p75NTRex-Fc fusion protein was used as antigen for panning. The naïve human antibody gene libraries HAL4 (Kappa) and HAL7 (Lambda) were employed. HAL4/7 libraries were established based on the phagemid vector pHAL14 that expressed antibody fragments as pIII fusion proteins containing a His_6-tag and a myc-tag (Hust *et al.*, 2007b; Kirsch *et al.*, 2008; Schütte *et al.*, 2009).

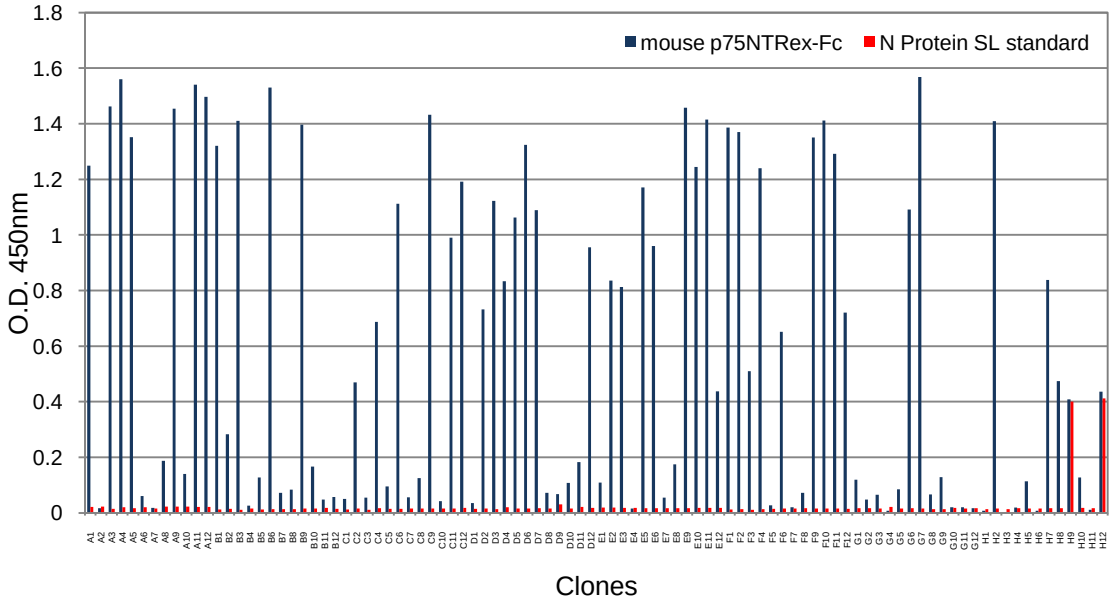

Figure 4.3 Identification of soluble monoclonal scFvs obtained from panning by ELISA. 100 ng of p75NTRex-Fc (shown in dark blue) and 100 ng of N protein SL standard (used as negative control, shown in red) were immobilized per well respectively. Soluble monoclonal scFvs were added after the antigen-coated plates were blocked with MPBST for 2 hr. Bound scFvs were detected using mAb anti-myc tag (9E10 hybridoma, 1:500) and goat anti-mouse IgG (Fab specific) HRP conjugated (1:5,000). Positions H9 and H12 were immobilized with 100 ng of lysozyme and incubated with anti-lysozyme scFv (SH102-10) served as positive controls.

In order to avoid isolating scFvs against human IgG1-Fc portion, N protein standard SL (Dade Benring, Germany) was used as competitor during panning procedure. The panning procedure was kindly performed by Saskia Helmsing (Institute for biochemistry and biotechnology, TU Braunschweig). After three rounds of panning and selection, single clones were isolated. Soluble monoclonal scFvs were produced in *E. coli* cultured in microtiter plates and assessed for their binding abilities to the immobilized p75NTRex-Fc fusion protein by ELISA (Fig. 4.3). ScFvs that bound to the p75NTRex-Fc fusion protein but not N protein SL standard were considered as positive binders (signal:noise ratios were greater than 40).

Out of the positive binders, three unique p75NTR-specific scFvs were finally isolated from the human antibody gene library HAL4/7 after DNA sequencing (Tab. 4.1). These scFvs were named as SH325-A11, SH325-B6, and SH325-G7. Their sequences were analyzed based on the integrative database of germline variable genes from the immunoglobulin loci of human (VBASE2). The VH of all three antibodies consisted of a VH3 germline gene. The variable light chains were only lambda chains in all three cases, but the V genes were derived from different germline genes.

Table 4.1 P75NTR-specific scFvs isolated by phage display.

Antibody clone	VH			VL	
	V	D	J	V	J
SH325-A11	IGHV3-23*01	IGHD6-13*01	IGHJ4*02	IGLV6	IGLJ3*02
SH325-B6	IGHV3	IGHD6-19*01	IGHJ4*02	IGLV3-21*02	IGLJ3*01
SH325-G7	IGHV3	IGHD2-15*01	IGHJ3*02	IGLV1-44*01	IGLJ3*02

The names of gene segments are given based on VBASE2. V: variable gene; D: diversity gene segment; J: joining gene segment.

4.3 Characterization of the p75NTR-specific scFvs

For the efficient production of the p75NTR-specific scFvs, the scFv cDNA sequences were constructed into the *E. coli* expression vector pOPE101-XP (Hust *et al.*, 2007a). The expression vector of pOPE101-XP was modified from pOPE101-215yol (Schmiedl *et al.*, 2000) and contained a myc tag and a His_6-tag for detection and purification. The p75NTR-specific scFvs were produced by periplasmic expression in *E. coli* and purified using IMAC. The concentrations of the purified scFvs were determined by SDS-PAGE (Fig. 4.4) in comparison with serial diluted scFv standard TM44 C7.3 (kindly provided by Dr. Torsten Meyer, Institute for Biochemistry and Biotechnology, TU Braunschweig). The concentrations were calculated as 12 µg/mL, 12 µg/mL and 11 µg/mL for SH325-A11, SH325-B6 and SH325-G7, respectively.

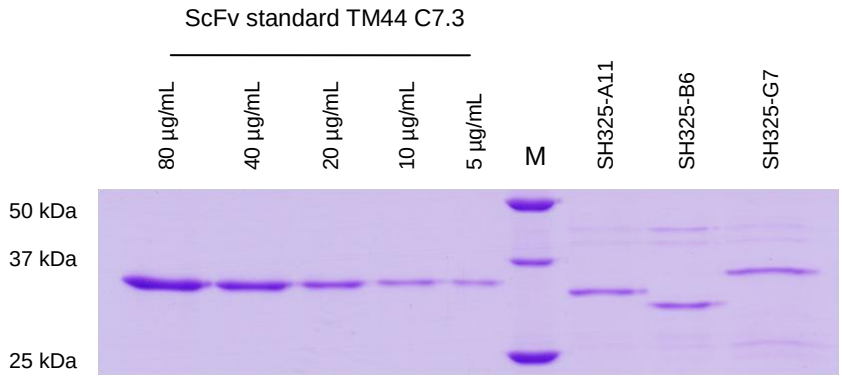

Figure 4.4 Concentration determination for the purified p75NTR-specific scFvs by SDS-PAGE. Serial diluted scFv standard TM44 C7.3 and purified p75NTR-specific scFvs were separated by reducing SDS-PAGE and stained with coomassie blue. M stands for protein marker.

4.3.1 The p75NTR-specific scFvs do not cross react with other neuronal surface proteins

Potential cross-reactivities of the p75NTR-specific scFvs with human IgG1 Fc domain and other neuronal surface proteins were investigated by antigen binding ELISA. In addition to N protein SL standard and BSA, five different human IgG1 Fc fusion proteins were used as negative controls, including rat TrkAex-Fc, rat TrkBex-Fc, mouse NgRex-Fc, mouse and human APPex-Fc. These recombinant proteins contained the extracellular portions of different neuronal proteins that are normally expressed by neurons. Moreover, the human IgG1 Fc domain used in these recombinant proteins is identical to that in p75NTRex-Fc.

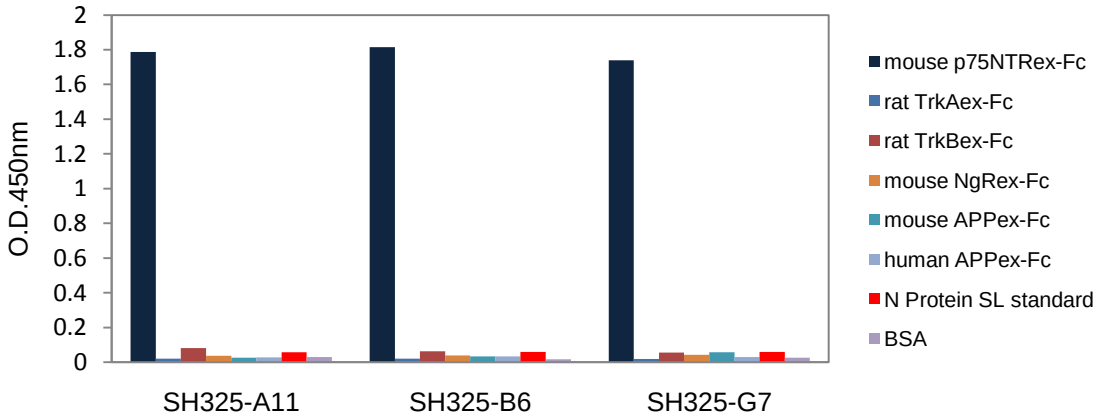

Figure 4.5 Cross-reactivities of the p75NTR-specific scFvs were determined by antigen binding ELISA. 100 ng of mouse p75NTRex-Fc (shown in dark blue), rat TrkAex-Fc, rat TrkBex-Fc, mouse NgRex-Fc, mouse or human APPex-Fc, N protein SL standard or BSA were immobilized in the plate for each well. The purified scFvs were added after the antigen-coated plates were blocked with

MPBST for 2 hr. Bound scFvs were detected using mAb anti-myc tag (9E10 hybridoma, 1:500) and goat anti-mouse IgG (Fab specific) HRP conjugated (1:5,000).

4.3.2 The p75NTR-specific scFvs bind to p75NTR with nanomolar affinities

Antibody affinity is essential for its application both *in vitro* and *in vivo*. Therefore, binding kinetics of the p75NTR-specific scFvs to the solid phase immobilized p75NTRex-Fc fusion protein was determined by SPR (Fig. 4.6).

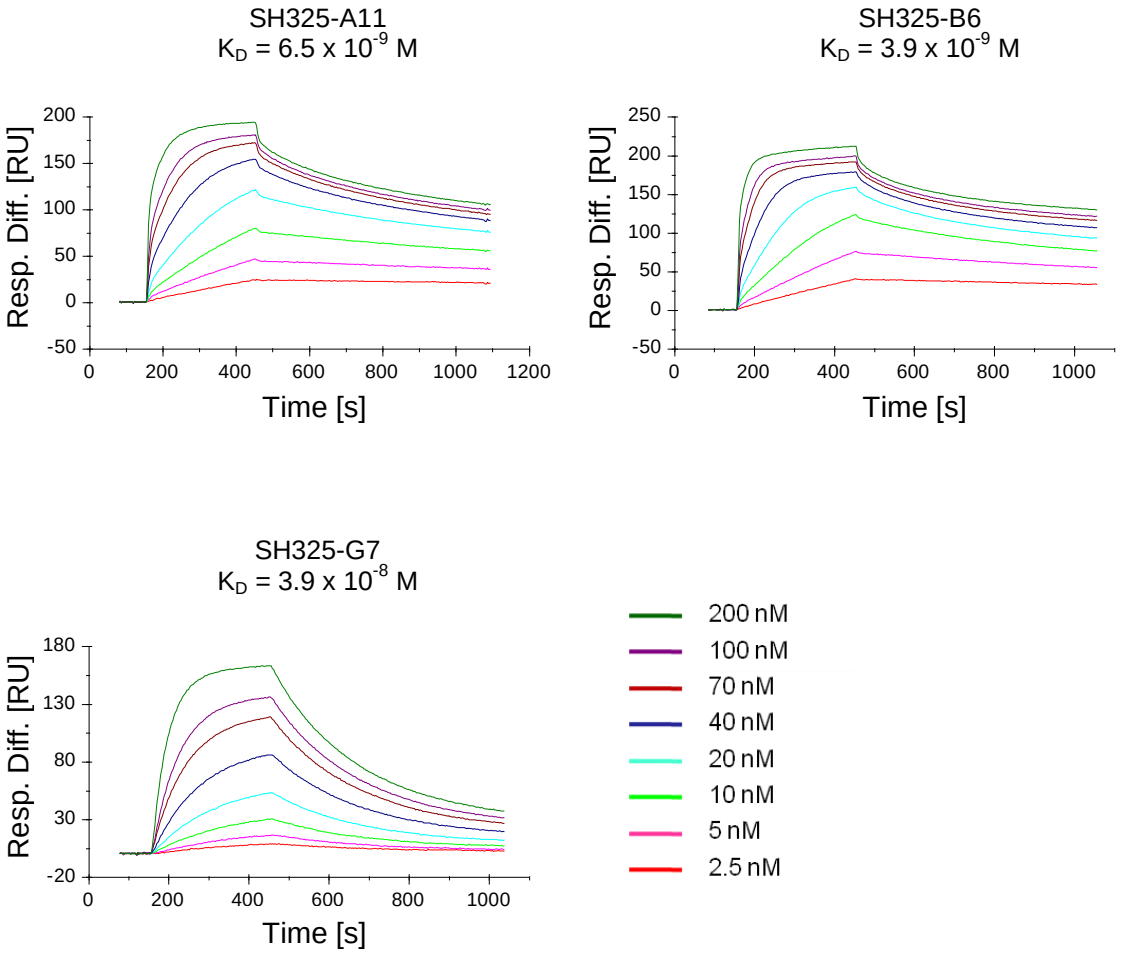

Figure 4.6 Determination of binding kinetics of the p75NTR-specific scFvs by SPR. The antigen p75NTRex-Fc and control protein rat TrkAex-Fc were separately coupled on flow cells via amine coupling. The p75NTR-specific scFvs with the concentrations from 2.5 nM to 200 nM were injected with an association time of 5 min and a disassociation time of 10 min. Curve fittings were done using 1:1 binding with drifting baseline. Mass transfer limitations were not present at this flow rate of 30 µL/min.

Recombinant rat TrkAex-Fc fusion protein was coupled on flow cells as the control to the p75NTRex-Fc. The binding experiment was performed at the flow rate of 30 µL/min using

peroxidase-free PBST and mass transfer limitations were not present at that flow rate. The p75NTR-specific scFvs with various concentrations from 2.5 nM to 200 nM were injected with an association time of 5 min and a dissociation time of 10 min. The affinities were detected in the range of nanomolar for all scFvs and the highest affinity was shown by SH325-A11 (see Tab. 4.2).

Table 4.2 Binding kinetics of the p75NTR-specific scFvs (k_a, k_d, K_D) determined by surface plasmon resonance.

	K_a (1/M•s)	k_d (1/s)	K_D (M)	Rmax (RU)	X^2
SH325-A11	2.44×10^5	1.57×10^{-3}	6.5×10^{-9}	156	2.31
SH325-B6	4.45×10^5	1.74×10^{-3}	3.9×10^{-9}	162	4.43
SH325-G7	1.08×10^5	4.22×10^{-3}	3.9×10^{-8}	180	0.24

4.3.3 Native p75NTR can be recognized by the p75NTR-specific scFvs

The p75NTR-specific scFvs need to neutralize p75NTR in the ER of living cells in this study. Therefore, it is crucial to verify that the p75NTR-specific scFvs bind to native p75NTR, in addition to the recombinant p75NTR immobilized on solid phase.

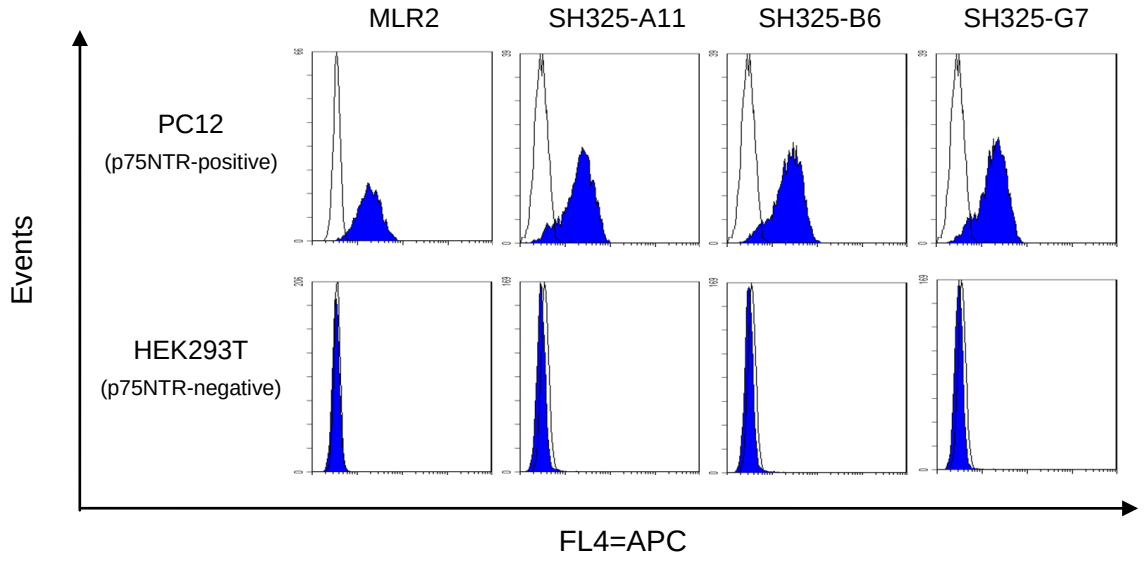

Figure 4.7 The p75NTR-specific scFvs recognize native p75NTR on PC12 cell surface. PC12 or HEK293T cells were stained with 250 ng of the p75NTR-specific scFvs (SH325-A11, SH325-B6, SH325-G7). Bound scFvs were detected by mAb mouse anti-His_6 (1:100) followed by goat anti-mouse IgG (Fc_γ specific) $F(ab')^2$ fragment APC conjugated (1:200). The p75NTR surface expression on PC12 cells was determined by staining PC12 and HEK293T cells with the mAb mouse anti-p75NTR (MLR2, 1:200) followed by goat anti-mouse IgG (Fc_γ specific) $F(ab')^2$ fragment APC

conjugated (1:200). The blue histograms represent the mAb mouse anti-p75NTR (MLR2) or the p75NTR-specific scFvs staining PC12 cells (upper row) and HEK 293T cells (lower row). The white histograms represent the controls stained with goat anti-mouse IgG (Fc_γ specific) $F(ab')^2$ fragment APC conjugated alone (MLR2 line) or *α*phOx scFv (rest lines) followed by mAb mouse anti-His_6 and goat anti-mouse IgG (Fc_γ specific) $F(ab')^2$ fragment APC conjugated.

Rat pheochromocytoma PC12 cells are neuron-like cells that endogenously express p75NTR (Ip *et al.*, 1993). The p75NTR expression on the PC12 cell surface was proved using the commercial mAb mouse anti-p75NTR (MLR2). Equal amount of cells were incubated with the p75NTR-specific scFvs and bound scFvs were analyzed by detecting the His_6-tag in flow cytometry (Fig. 4.7). HEK293T cells were used as negative controls that do not express p75NTR. An *α*phOx scFv was used as the comparable control to the p75NTR-specific scFvs, because the hapten phOx is not present in either PC12 or HEK293T cells. All the p75NTR-specific scFvs bound to PC12 cells, while none of them showed obvious bindings to HEK 293T cells compared to the samples stained by the *α*phOx scFv.

4.4 No steric interference between mAb mouse anti-p75NTR (MLR2) and the p75NTR-specific scFvs

In order to examine p75NTR surface knockdown by an ER-intrabodies, p75NTR needs to be detected through a non-overlapping epitope besides those interacted with ER-intrabodies. A competition ELISA was performed between mAb mouse anti-p75NTR (MLR2) and the p75NTR-specific scFvs (Fig. 4.8).

Signal intensities of three p75NTR-specific scFvs were enhanced according to the concentration increase (Fig. 4.8 red lines). Coated p75NTR-Fc fusion proteins were saturated as the scFv concentration was greater than 5.0 µg/mL, since the signal intensities from the scFvs reached to a plateau at that concentration. In parallel, nearly constant signal intensities were obtained from mAb mouse anti-p75NTR (MLR2) in spite of the variations of scFv concentrations (Fig. 4.8 blue lines). There were practically no competitions between the p75NTR-specific scFvs and mAb mouse anti-p75NTR (MLR2). Therefore, the binding of mAb mouse anti-p75NTR (MLR2) to p75NTR was hardly interfered by the p75NTR-specific scFvs. The HRP conjugated secondary antibodies did not show any non-specific binding according to the determination of negative controls (data not shown).

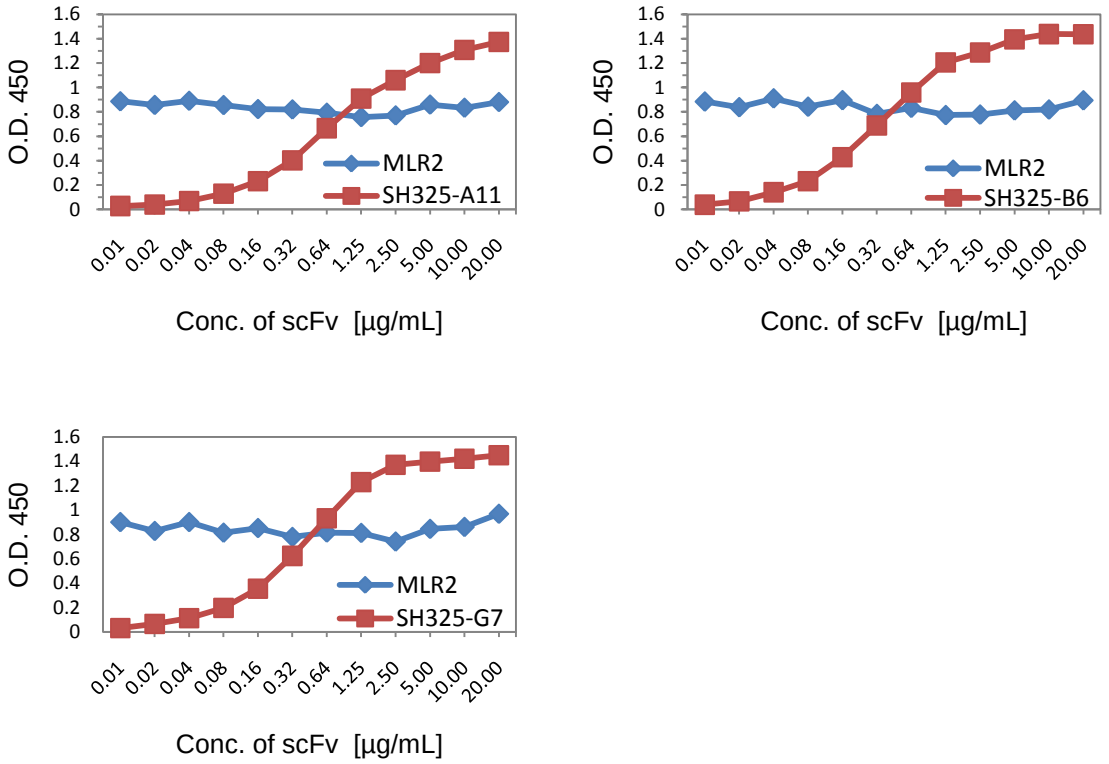

Figure 4.8 Competition ELISA between mAb mouse anti-p75NTR (MLR2) and the p75NTR-specific scFvs. 100 ng of mouse p75NTRex-Fc fusion protein was immobilized in BD plates for each well. Serial diluted p75NTR-specific scFvs were added and incubated for 1.5 hr at 37 °C. After 3x washing with PBST, the plates were again incubated with mAb mouse anti-p75NTR (MLR2, 1:5,000) for 1.5 hr at 37 °C. The plates were 3x washed with PBST. Bound scFvs and mAb mouse anti-p75NTR (MLR2) were detected by mAb mouse anti Penta-His HRP conjugated (1:5,000) and pAb goat anti-mouse IgG (Fc specific) HRP conjugated (1:5,000) in corresponding plates respectively.

4.5 Surface knockdown of p75NTR in PC12 cells by the p75NTR-specific ER-intrabodies

The sequences of the p75NTR-specific scFvs were subcloned into the bicistronic knockdown vectors (Fig. 3.2) to generate ER-intrabodies (SH325-A11-KDEL, SH325-B6-KDEL and SH325-G7-KDEL). P75NTR surface knockdown was firstly determined in PC12 cells. PC12 cells were transiently transfected with the bicistronic knockdown vectors. The ER-intrabody αphOx-KDEL was used as the control which contained the αphOx scFv fusing to the ER retention signal KDEL. Four days after transfection, the p75NTR surface expression in PC12 cells was determined in flow cytometry (Fig. 4.9). Since the ER-intrabody and reporter EGFP-F were translated from a single transcript, it thus allowed a simple analysis of the cells expressing the ER-intrabodies by flow cytometry.

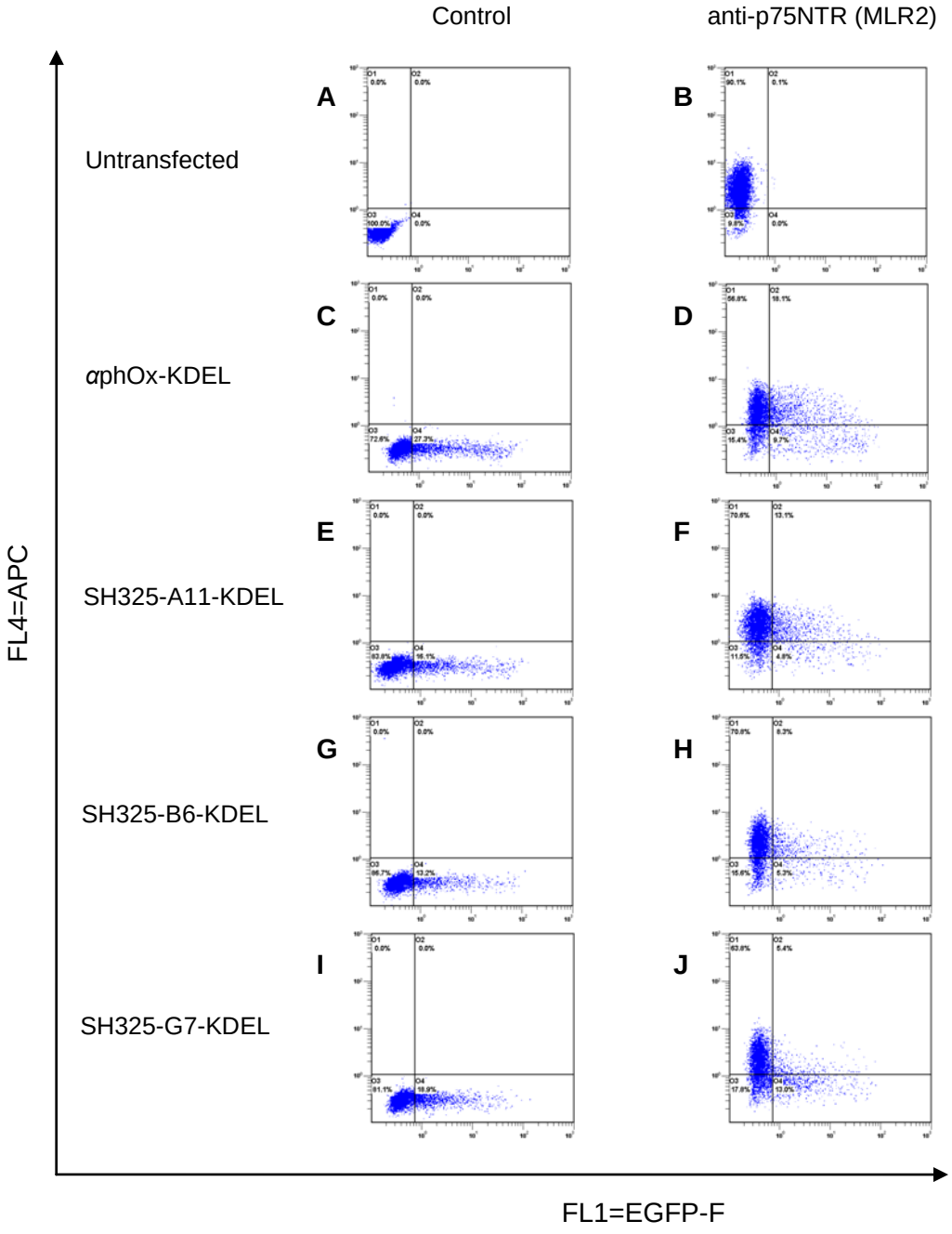

Figure 4.9 Surface knockdown of p75NTR in PC12 cells by the p75NTR-specific ER-intrabodies. PC12 cells were transiently transfected with the p75NTR-specific ER-intrabodies and *α*phOx-KDEL. Four days after transfection, the p75NTR surface expressions were determined with mAb mouse anti-p75NTR (MLR2, 1:200) followed by goat anti-mouse IgG (Fc_γ specific) $F(ab')^2$ fragment APC conjugated (1:200, B, D, F, H, and J). Cells stained with secondary antibody alone were served as control (A, C, E, G, and I). FL1 stands for the transfected cells by showing the EGFP-F expression. FL4 stands for the p75NTR surface expression by illustrating the APC fluorescence.

The untransfected PC12 cells showed the p75NTR expressed on their cell surfaces (Fig. 4.9B). Cells expressing the ER-intrabodies were located in the upper and lower right

quadrants of the dot plots because of the EGFP-F fluorescence (Fig. 4.9D, F, H, and J). Cell population was expected to be shifted from the upper right quadrant to the lower right quadrant, if the p75NTR surface expression was downregulated by the p75NTR-specific ER-intrabodies. There were high levels of p75NTR expressed on the surfaces of the cells transfected with *α*phOx-KDEL (Fig. 4.9D). No considerable reduction of the p75NTR surface expression could be detected in the SH325-A11-KDEL transfected cells (Fig. 4.9F). In the cells transfected with SH325-B6-KDEL, a moderate decrease of the p75NTR surface expression was measured (Fig. 4.9H). However, the p75NTR surface expression was remarkably inhibited in the SH325-G7-KDEL transfected cells, resulting in a shift of the transfected cell population from the $FL4^+$ quadrant to the $FL4^-$ quadrant (Fig. 4.9J). The unspecific bindings were excluded by staining cells with secondary antibody alone (Fig. 4.9A, C, E, G, and I).

To analyze the effects of the p75NTR-specific ER-intrabodies in more detail, an overlay analysis was performed between the cells expressing the p75NTR-specific ER-intrabodies and those expressing the control *α*phOx-KDEL (Fig. 4.10).

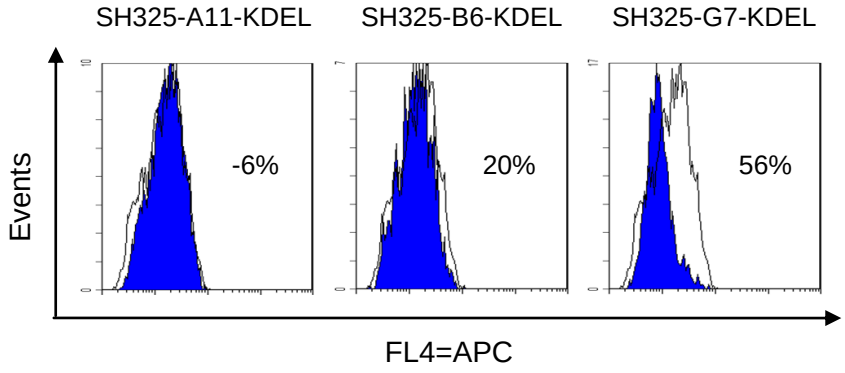

Figure 4.10 Overlay analysis for the p75NTR surface knockdown in PC12 cells. The blue histograms represent the p75NTR surface expression of the PC12 cells expressing the p75NTR-specific ER-intrabodies (SH325-A11-KDEL, SH325-B6-KDEL and SH325-G7-KDEL). The white histograms represent the p75NTR surface expression of the PC12 cells expressing the control *α*phOx-KDEL. FL4 stands for the p75NTR surface expression by illustrating the APC signal intensity. The p75NTR surface knockdown ratios were indicated in percent.

P75NTR knockdown ratios were calculated by comparing the mean value of APC signal intensity, i.e. the p75NTR surface expression level, between the cells expressing the p75NTR-specific ER-intrabodies and the cells expressing the control *α*phOx-KDEL. The p75NTR knockdown ratios were showed in percent. There was no decrease in the p75NTR surface levels in the cells expressing SH325-A11-KDEL (-6% reduction) compared to *α*phOx-KDEL, whereas a slight effect was obtained from the cells expressing SH325-B6-KDEL (20%

reduction). The most considerable effect was acquired in the PC12 cells transfected with the SH325-G7-KDEL construct. Approximately 56% of surface directed p75NTR was inhibited from surface translocation by this ER-intrabody.

4.6 Surface knockdown p75NTR in NSC19 cells by the p75NTR-specific ER-intrabodies

Besides PC12 cells, the effect of p75NTR-specific ER-intrabodies was also identified in NSC19 motor neuron cells. NSC19 was generated by fusing the mouse neuroblastoma N18TG2 cells with motor neuron-enriched embryonic day 12–14 mouse spinal cord cells (Cashman *et al.*, 1992). It was validated to express p75NTR on cell surface. Four days after transfection, NSC19 cells were stained against p75NTR and subjected to flow cytometric analysis. An overlay analysis was carried out between the cells transfected with the p75NTR-specific ER-intrabodies and those transfected with *α*phOx-KDEL ER-intrabody (Fig. 4.11).

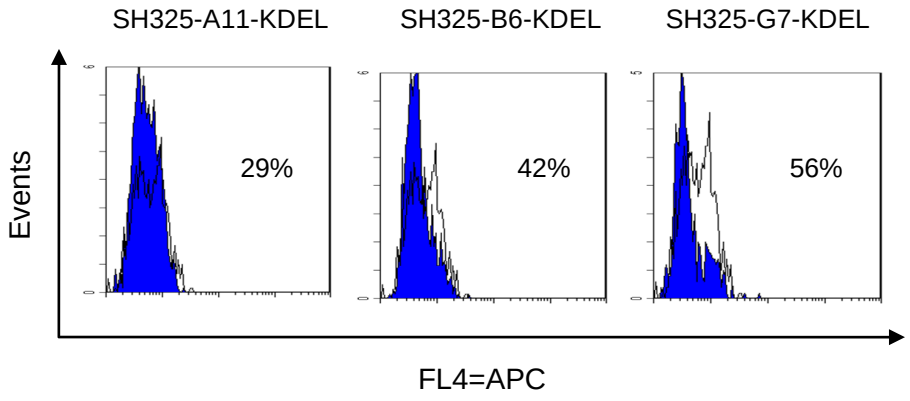

Figure 4.11 Surface knockdown of p75NTR in NSC19 cells by the p75NTR-specific ER-intrabodies. NSC19 cells were transiently transfected with the p75NTR-specific ER-intrabodies and the control *α*phOx-KDEL. Four days after transfection, the p75NTR surface expressions were determined with mAb mouse anti-p75NTR (MLR2, 1:200) followed by goat anti-mouse IgG (Fc_γ specific) $F(ab')^2$ fragment APC conjugated (1:200). Unspecific bindings were excluded by staining cells with secondary antibody alone (data not shown). The blue histograms represent the p75NTR surface expressions of the NSC19 cells expressing the p75NTR-specific ER-intrabodies (SH325-A11-KDEL, SH325-B6-KDEL and SH325-G7-KDEL). The white histograms represent the p75NTR surface expressions of the NSC19 cells expressing the control *α*phOx-KDEL. FL4 stands for the p75NTR surface expression by illustrating the APC signal intensity. The p75NTR surface knockdown ratios were indicated in percent.

P75NTR surface knockdown ratios were calculated and the effects of the p75NTR-specific ER-intrabodies in NSC19 cells showed nearly the same potency on the p75NTR surface expression levels as in PC12 cells. The maximal knockdown ratio was obtained from SH325-G7-KDEL (56% reduction) as it exhibited in PC12 cells. However, the SH325-A11-KDEL or

SH325-B6-KDEL transfected cells showed 29% or 42% reduction on the p75NTR surface expression respectively, which were enhanced in comparison with the results from PC12 cells.

4.7 The p75NTR-specific ER-intrabodies are expressed in different levels in PC12 cells

It showed that only the ER-intrabody SH325-G7-KDEL had the obvious effect on downregulating the p75NTR surface expression. The ER-intrabody intracellular expressions were analyzed to address this question. The knockdown vectors encoding the p75NTR-specific ER-intrabodies were transiently transfected into PC12 cells. Transfection efficiencies were determined in flow cytometry. Four days after transfection, 2 x 10^6 cells from each sample were lysed in 200 µL of lysis buffer. The intracellular expression of the p75NTR-specific ER-intrabodies was evaluated by immunoblotting (Fig. 4.12A). The negative control resulted from the cell extracts of untransfected PC12 cells.

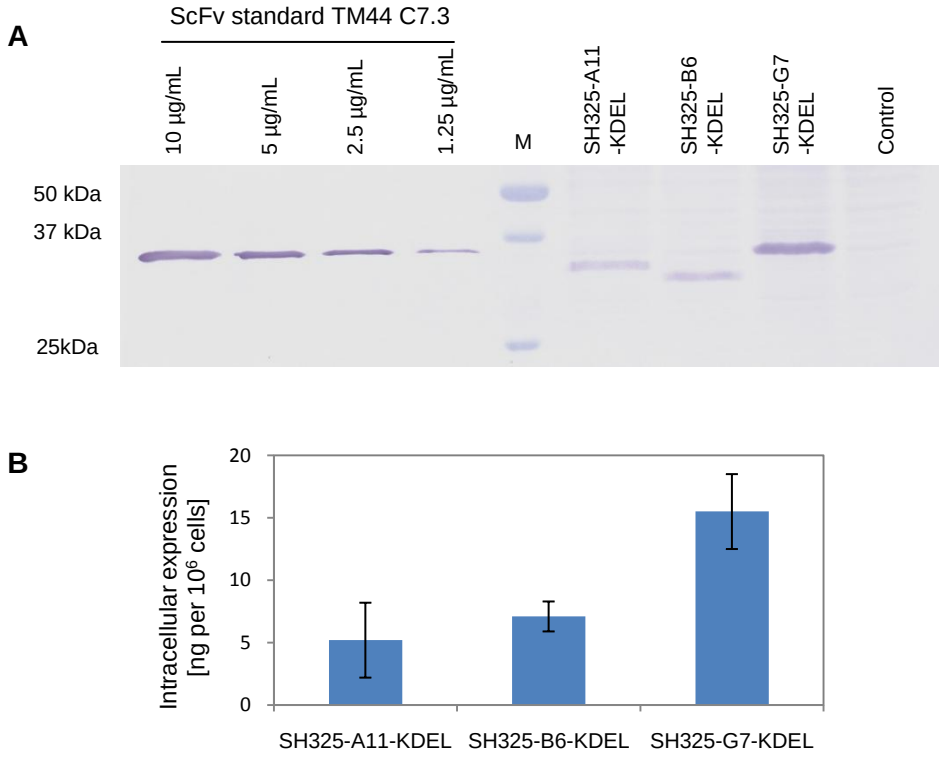

Figure 4.12 Intracellular expressions of the p75NTR-specific ER-intrabodies in PC12 cells. (A) Quantitative determination by immunoblotting. PC12 cells were transiently transfected with the constructs encoding the p75NTR-specific ER-intrabodies. Four days after transfection, cell extracts were prepared and separated by SDS-PAGE. After immunoblotting, the membrane was incubated with mouse anti-His_5 (1:2,000) for 1 hr at RT and followed by goat anti-mouse IgG (Fab-specific) AP conjugated (1:5,000) for 1 hr at RT. The control resulted from the cell extracts of untransfected PC12 cells. M stands for protein marker. Intracellular expression levels of the p75NTR-specfic ER-intrabodies were calculated according to the scFv standard TM44 C7.3. (B) Intracellular expression

levels varied among the p75NTR-specific ER-intrabodies. The error bars were calculated from three time-independent experiments.

The signal intensities were analyzed by ImageJ software. According to the calibration curve obtained from the scFv standard TM44 C7.3, the intracellular expressions of these p75NTR-specific ER-intrabodies were quantitated (Fig. 4.12B). As a result, the intracellular expression levels of SH325-A11-KDEL (5.2 ng per 10^6 cells) and SH325-B6-KDEL (7.1 ng per 10^6 cells) were approximately as half as that of SH325-G7-KDEL (15.5 ng per 10^6 cells) in transiently transfected PC12 cells.

4.8 P75NTR surface expression has been suppressed by the ER-intrabody (SH325-G7-KDEL) for more than 8 days in PC12 cells

To analyze the kinetics of the p75NTR-specific ER-intrabody effect, a time course experiment of p75NTR knockdown over eight days was performed using the construct of SH325-G7-KDEL. PC12 cells were transiently transfected with SH325-G7-KDEL and *α*phOx-KDEL, respectively. Samples were obtained at different time points and the transfected cells were analyzed in flow cytometry. The results were shown in Fig. 4.13.

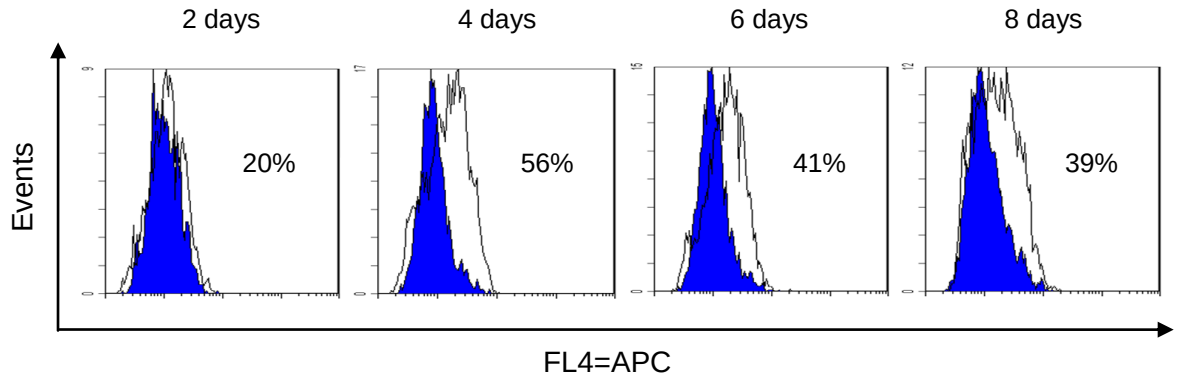

Figure 4.13 Kinetics of the p75NTR-specific ER-intrabody (SH325-G7-KDEL) effect in PC12 cells. PC12 cells were transiently transfected with the p75NTR-specific ER-intrabody and the control *α*phOx-KDEL. Cells were harvested at different time points. The p75NTR surface expressions were determined with mAb mouse anti-p75NTR (MLR2, 1:200) followed by goat anti-mouse IgG (Fc_γ specific) $F(ab')^2$ fragment APC conjugated (1:200). The blue histograms represent the p75NTR surface expressions of the PC12 cells expressing SH325-G7-KDEL. The white histograms represent the p75NTR surface expressions of the PC12 cells expressing *α*phOx-KDEL. FL4 stands for the p75NTR surface expression by illustrating the APC signal intensity. The p75NTR surface knockdown ratios were indicated in percent.

The p75NTR-specific ER-intrabody effect progressively increased from two to four days after transfection with SH325-G7-KDEL, and the maximal knockdown ratio (56% reduction) appeared at four days post-transfection. Afterwards, the surface expression level of p75NTR

was still suppressed in PC12 cells, although a slight increase of p75NTR surface expression was observed.

4.9 Knockdown p75NTR by the ER-intrabody (SH325-G7-KDEL) does not activate the unfolded protein response (UPR)

In order to assess the impact of the p75NTR knockdown via the ER-intrabody, it was further determined whether the UPR was activated during the knockdown process up to 8 days. An ER resident 94 kDa glucose-regulated protein (GRP94) was used as reporter protein for ER stress response. Its expression is upregulated due to ER stress (Bando *et al.*, 2003). PC12 cells were transiently transfected with SH325-G7-KDEL. Cells from different time points (2-8 days) were sorted by BD FACSAria™ II based on EGFP-F fluorescence, i.e. the p75NTR-specific ER-intrabody expression. The sorting procedure was kindly performed by Dr. Lothar Groebe (Experimental Immunology, Helmholtz-Zentrum für Infektionsforschung (HZI), Germany). The possible influence of sorting efficiency on the result was extinguished since it was determined as about 60% for all samples. Negative and positive controls were prepared by treating PC12 cells with DMSO alone or 20 µg/mL of tunicamycin for 24 hr. Tunicamycin is known as an UPR stimulus because it inhibits N-linked glycosylation (Mahoney and Duksin, 1979) and influences glycoprotein membrane transports (Olden *et al.*, 1979). The expression levels of GRP94 were evaluated by immunoblotting (Fig. 4.14). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control to ensure equal protein loading and was detected on the same membrane.

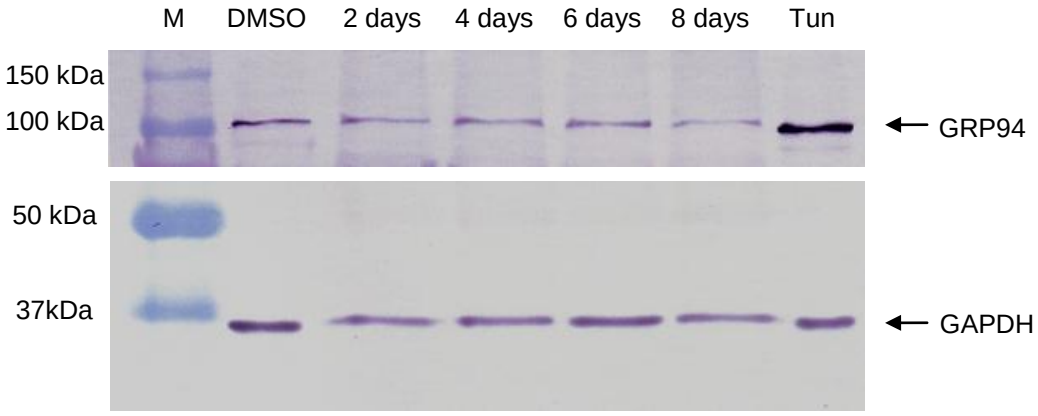

Figure 4.14 Knockdown p75NTR by the ER-intrabody (SH325-G7-KDEL) does not activate the UPR. The SH325-G7-KDEL transfected PC12 cells from different time points (2-8 days) were sorted by BD FACSAria™ II based on the EGFP-F fluorescence. Cells treated with 20 µg/mL of tunicamycin (Tun) or solvent alone (DMSO) were used as the positive or negative control, respectively. Cell extracts were prepared and blotted onto a PVDF membrane. The membrane was incubated with rabbit anti-GRP94 (1:1,000) and rabbit anti-GAPDH (1:5,000) for 1 hr at RT. After 3x washing with PBST, the membrane was subsequently incubated with goat anti-rabbit IgG AP conjugated antibody

(1:5,000) for 1 hr at RT. GAPDH served as an internal control to ensure equal protein loading. M stands for protein marker.

None of the GRP94 expression levels from different time point samples showed detectable upregulation compared to the DMSO treated negative control. In contrast, tunicamycin treated cells showed a considerable increase in the GRP94 expression.

4.10 Suppressing p75NTR surface expression by the ER-intrabody (SH325-G7-KDEL) impacts dendrite complexity in mouse hippocampal primary cultures

So far, the p75NTR surface knockdown has been proved in the cell lines by the p75NTR-specific ER-intrabody SH325-G7-KDEL. In order to evaluate the effect of this ER-intrabody in neurons, dissociated primary cultures of mouse hippocampal neurons were prepared using mice (C57 Bl/6) embryonic day E18. This work was done in collaboration with the institute for cellular neurobiology, TU Braunschweig (leaded by Prof. Martin Korte) and kindly helped by Dr. Marta Zagrebelsky and Janina Beuker.

Primary hippocampal cultures were transfected at 7 DIV (days *in vitro*) using Lipofectamine2000 with SH325-G7-KDEL, *α*phOx-KDEL or fGFP, which is a vector encoding EGFP-F. Four days after transfection, p75NTR surface expression was detected by pAb rabbit anti-human p75NTR (extracellular) and anti-rabbit antibody Cy3 conjugated. Neurons were fixed after immunostaining to avoid permeablization. To demonstrate that neurons were not permeabilizated during immunocytochemistry, pAb rabbit anti-human p75NTR against the intracellular domain of p75NTR was used (data not shown).

After immunostaining, transfected neurons were found either with p75NTR (p75NTR-positive) or without p75NTR (p75NTR-negative) surface expressions in all the samples (Fig. 4.15). Because the constructs of *α*phOx-KDEL and fGFP had no influences on p75NTR surface expression, the endogenously p75NTR-positive and -negative neurons were designated by transfected with these constructs.

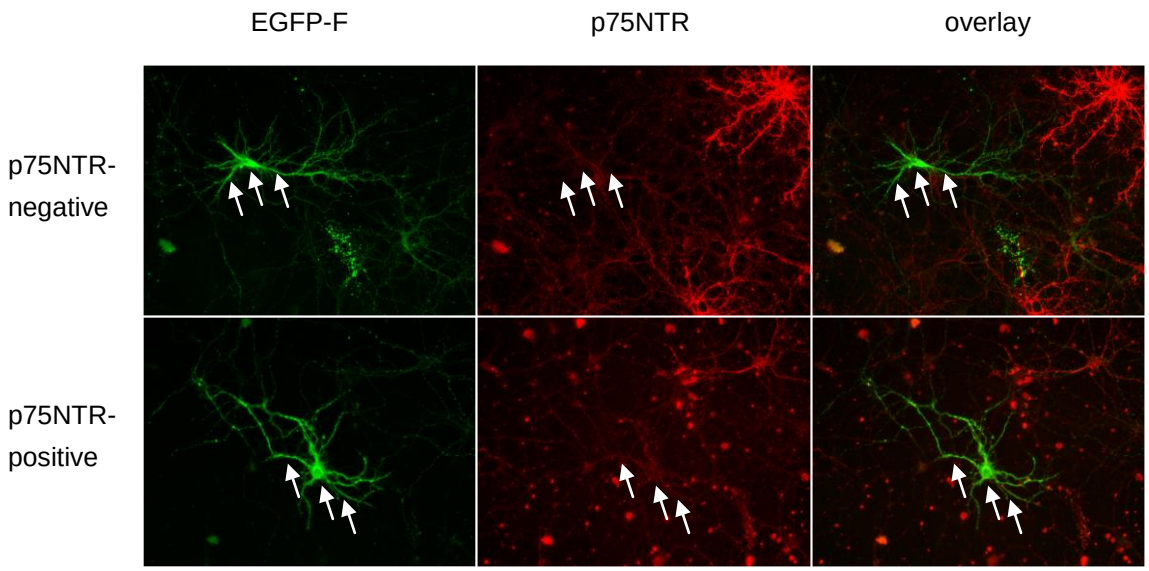

Figure 4.15 Examples of immunofluorescence images showing p75NTR-negative (upper row) and p75NTR-positive (lower row) neurons after transfection with SH325-G7-KDEL. Primary hippocampal cultures were transfected at 7 DIV (days *in vitro*) with SH325-G7-KDEL. Four days after transfection, p75NTR surface expression was detected by pAb rabbit anti-human p75NTR (extracellular, 1:1,000) and anti-rabbit antibody Cy3 conjugated (1:500). Arrows indicates the positions of cell somata and dendrites.

Due to the expression of EGFP-F, the entire dendritic trees of transfected neurons were intensely labeled and could be imaged using fluorescence microscopy. Only the pyramidal neurons were concerned in this experiment. Morphology of the transfected neurons was determined by Sholl analysis (Sholl, 1953). A significant increase of dendritic complexity was detected in the proximal region (40 µm from the cell soma, $p = 0.04$) of the SH325-G7-KDEL transfected p75NTR-negative neurons in comparison with the p75NTR-positive neurons (Fig. 4.16A). Furthermore, in comparison with the *α*phOx-KDEL or fGFP transfected p75NTR-positive neurons, the number of dendrite crossings significantly increased in the SH325-G7-KDEL transfected p75NTR-negative neurons (Fig. 4.16B). The significant differences were illustrated in the proximal region of a distance about 40-70 µm from the cell soma ($p < 0.05$). In addition, no significant alterations were found between p75NTR-positive or -negative neurons that were transfected with *α*phOx-KDEL or fGFP (Fig. 4.16C, D).

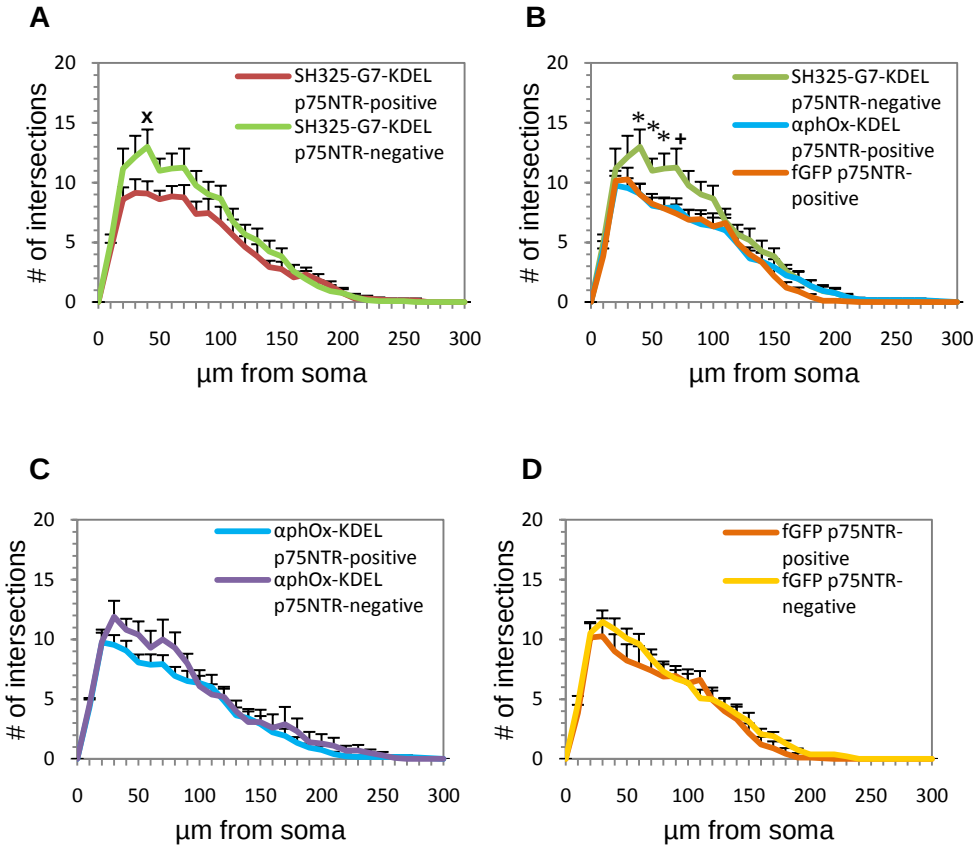

Figure 4.16 Sholl analysis for dendritic complexity of the p75NTR-positive and p75NTR-negative neurons transfected with SH325-G7-KDEL, αphOx-KDEL or fGFP. (A) Analysis for dendrite complexity between SH325-G7-KDEL transfected p75NTR-positive (n=13) and SH325-G7-KDEL transfected p75NTR-negative (n=12) neurons. (B) Analysis for dendrite complexity among SH325-G7-KDEL transfected p75NTR-negative (n=12), *α*phOx-KDEL transfected p75NTR-positive (n=17) and fGFP transfected p75NTR-positive (n=18) neurons. (C) Analysis for dendrite complexity between *α*phOx-KDEL transfected p75NTR-positive (n=17) and *α*phOx-KDEL transfected p75NTR-negative (n=10) neurons. (D) Analysis for dendrite complexity between fGFP transfected p75NTR-positive (n=18) and fGFP transfected p75NTR-negative (n=13) neurons. Significant differences for Sholl analysis are indicated as follows: x, SH325-G7-KDEL transfected p75NTR-negative neurons are significantly different from SH325-G7-KDEL transfected p75NTR-positive neurons; *, SH325-G7-KDEL transfected p75NTR-negative neurons are significantly different from both *α*phOx-KDEL transfected p75NTR-positive and fGFP transfected p75NTR-positive neurons; +, SH325-G7-KDEL transfected p75NTR-negative neurons are significantly different from fGFP transfected p75NTR-positive neurons (p <0.05). Error bars represent SEM.

5 Discussion

P75 neurotrophin receptor (p75NTR) is the first neurotrophin receptor that was isolated. It belongs to the tumor necrosis factor receptor (TNFR) superfamily according to the death domain in its cytoplasmic portion. Although p75NTR has been identified to be critical in many aspects on the function of vertebrate neurons, its exact physiological role is not yet fully understood (Lu *et al.*, 2005; Nykjaer *et al.*, 2005; Yamashita *et al.*, 2005). P75NTR normally has to collaborate with many different protein partners because of the lack of intrinsic kinase signaling in its intracellular region and the functions of p75NTR strongly depending on the molecular and cellular context (Hempstead, 2002). It mediates neurite outgrowth, cell apoptosis as well as cell survival (Dechant and Barde, 1997; Yoon *et al.*, 1998; Mi *et al.*, 2004; Nykjaer *et al.*, 2004; Sole *et al.*, 2004). Recent works also indicate that p75NTR regulates neurogenesis and long-term depression (LTD) in mouse hippocampus and further implicate that p75NTR plays an essential role in learning and memory formation in brain (Rosch *et al.*, 2005; Woo *et al.*, 2005; Catts *et al.*, 2008).

So far, most functional studies of p75NTR have been employing gene knockout $p75NTR^{-/-}$ mice. However, gene-targeted knockout is a time-consuming work on one hand, and alternative splicing products, on the other hand, have been reported in both $p75NTR^{exonIII}$ and $p75NTR^{exonIV}$ knockout phenotypes (von Schack *et al.*, 2001; Paul *et al.*, 2004). These alternative splicing products are suggested to be proapoptotic fragments since both of them contain the intact intracellular domain of p75NTR. This postulation was proved by the recent work. Overexpression of the p75NTR intracellular domain in mice led to increased neuronal death in both the central and peripheral nervous system (Majdan *et al.*, 1997). In addition, p75NTR has been shown to undergo α- and then γ-secretase intramembrane proteolytic cleavage releasing its intracellular C-terminal fragment (Jung *et al.*, 2003; Kanning *et al.*, 2003). The translocation of the p75NTR intracellular fragment derived from γ-secretase processing to the nucleus (Kanning *et al.*, 2003) implies that it may act as an important role in nuclear signaling.

Besides gene-target knockout, several knockdown techniques have been applied to inhibit specific protein functions, such as siRNAs, RNA apatmers and ER-intrabodies (Mocellin and Provenzano, 2004; Boldicke, 2007; Yan and Levy, 2009). Among these approaches, ER-intrabodies trap specific targets within the ER by constructing an ER retention signal at C-terminal. In contrast to the gene-targeted knockout or RNAi technology that silence gene expression, ER-intrabodies provide the possibility to neutralize the functions of specific secretory proteins at the post-translational level and thus avoid the probable generation of abnormal splicing products. Besides high affinity and specificity, ER-intrabodies are very stably expressed in mammalian cells compared to siRNAs (Fish and Kruithof, 2004) or aptamers (Famulok *et al.*, 2000). Moreover, siRNAs have been identified to activate an

interferon-mediated activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway, resulting in unknown effects in target cells (Sledz *et al.*, 2003). Whereas intrabody may prove be less immunogenic than other therapeutics and thereby suggests it can be potentially useful in the therapeutical applications, especially in neurodegenerative diseases (Kontermann, 2004; Miller and Messer, 2005). To date, ER-intrabody knockdown technology is becoming one of potent tools to downregulate target protein function.

ScFvs become the most use in intrabody applications in the past decade. Because of the small size, scFvs are easily expressed and assembled as functional molecules. Many techniques have been developed for scFv screening, and phage display panning has become the most robust, versatile and widespread method over the past 10 years (Hust *et al.*, 2007b). Based on antibody libraries, recombinant scFvs specific to nearly any target antigen can be isolated *in vitro* at high throughput in a relative short period. In this work, antibody fragments in scFv formats were isolated by phage display against the recombinant antigen of p75NTRex-Fc. The antigen p75NTRex-Fc was produced by fusing the extracellular domain of mouse p75NTR with the human IgG1 Fc domain to improve secretory expression in mammalian cells and facilitate purification by protein A. The purity and size of p75NTRex-Fc were demonstrated by immunoblotting. As a consequence of the glycosylations of p75NTR (Gong *et al.*, 2008) and IgG1 Fc domain (Lund *et al.*, 1996; Jefferis *et al.*, 1998), purified p75NTRex-Fc fusion protein migrated to ca. 100 kDa on the membrane.

Three scFvs, SH325-A11, SH325-B6 and SH325-G7, against the extracellular domain of mouse p75NTR were selected from the naïve human antibody gene libraries HAL4 (Kappa) and HAL7 (Lambda) after three panning rounds (Hust *et al.*, 2007b; Kirsch *et al.*, 2008; Schütte *et al.*, 2009). The p75NTR-specific scFvs were selected against the recombinant p75NTRex-Fc directly immobilized onto the plastic surface in immunostripes in panning procedure. The p75NTR-specific scFvs were produced by periplasmic expression in *E. coli* and purified using IMAC.

It was important that the candidate scFvs were not cross-reactive with other neuronal receptors, such as TrkA and TrkB, which were related but probably antagonistically functional to p75NTR. In siRNA knockdown technique, the specificity of siRNA is continually argued due to its off-target effects (Couzin, 2004). All selected scFvs showed high specificities to p75NTR, but not other neuronal proteins used as controls in the antigen binding ELISA determination.

The affinities were determined for these p75NTR-specific scFvs by SPR. All the p75NTR-specific scFvs have the affinities in the range of naomolar, which are expected for isolated antibody fragments from naïve antibody gene library by phage display (Griffiths *et al.*, 1994).

Since the direct coating may lead protein into a partial denaturation, it is necessary to identify the native conformational p75NTR can be recognized by the scFvs selected against the directly immobilized recombinant p75NTR. In flow cytometric analysis, all the p75NTR-specific scFvs bound to PC12 cells that express p75NTR, whereas they did not bind to HEK293T cells that are negative for p75NTR. It is well known that the post-translational modifications are completed within the ER before they are transported to Golgi complex and secreted to the cell surface. Hence, the recognition of native p75NTR on the PC12 cell surface strongly indicates that the p75NTR-specific scFvs are also capable to associate with p75NTR in the ER.

In order to knockdown p75NTR, the p75NTR-specific scFvs were engineered with the C-terminal ER retention motif KDEL to generate the ER-intrabodies, SH325-A11-KDEL, SH325-B6-KDEL and SH325-G7-KDEL. Additionally, the His_6-tag sequence was inserted between the scFv and the KDEL allowing detection. The conformations of scFvs are supposed not to be changed with these motifs, since the His_6-tag and KDEL sequence have little effect on the native protein structure, and the scFv stability may increase in the presence of KDEL (Schouten *et al.*, 1996; Carson *et al.*, 2007). In order to transfer the ER-intrabodies into mammalian cells, a novel bicistronic knockdown vector was constructed (Fig 3.1). The first cistron ER-intrabody was mediated by a CMV promoter, while the EGFP-F was directed as the second cistron by a mutant EMCV IRES element in cap-independent mechanism. EGFP-F is a farnesylated form of EGFP that remains bound to the plasma membrane in both living and fixed cells. Because the ER-intrabody and EGFP-F are simultaneously translated from a single transcript, the intrabody-expressing cells can be easily distinguished in flow cytometry and fluorescence microscopy.

The effect of the p75NTR-specific ER-intrabodies on the p75NTR surface expression was firstly demonstrated in PC12 cells. Four days after transfection with the ER-intrabody constructs, the p75NTR surface expressions were determined by a detection antibody, mAb mouse anti-p75NTR (MLR2). As shown in the competition ELISA, there are no steric interference between mAb mouse anti-p75NTR (MLR2) and the p75NTR-specific scFvs. Hence, the downregulations of p75NTR surface expression levels can only be caused by the effect of the p75NTR-specific ER-intrabodies. In flow cytometric analysis, the p75NTR surface expression was only dramatically downregulated by SH325-G7-KDEL, which has the lowest affinity among the p75NTR-specific ER-intrabodies. This phenomenon was also found in p75NTR-expressing NSC19 cells. Although SH325-A11-KDEL and SH325-B6-KDEL showed enhanced p75NTR surface knockdown ratios in NSC19 cells compared to PC12 cells, the maximal knockdown ratio was obtained from SH325-G7-KDEL. Considering that SH325-G7-KDEL expressed much more strongly than SH325-A11-KDEL and SH325-B6-KDEL in transiently transfected PC12 cells, it seems that the intracellular expression amount of ER-intrabody is one of the pivotal factors that impact the effect on target protein knockdown. The enhanced effects of SH325-A11-KDEL and SH325-B6-KDEL in NSC19

cells might arise from the improvement of the ER-intrabody intracellular expressions. However, the knockdown ratio might not be strengthened when certain expression level was reached for an ER-intrabody, as shown by SH325-G7-KDEL. Improvement of the ER-intrabody affinity or avidity becomes a promising way to increase the knockdown ratio in that situation. In SH325-G7-KDEL transfected PC12 cells, the maximal p75NTR knockdown ratio (56% reduction) was calculated in comparison with the *α*phOx-KDEL transfected cells. It has illustrated that there were cells detected as p75NTR negative in all samples independent on the knockdown construct transfer (Fig. 4.9B, D, F, H, and J). This is probably the reason that the knockdown ratio could not be calculated as high as the former works in which the cells were engineered for target overexpression.

A time course experiment was performed using the construct of SH325-G7-KDEL to analyze the kinetics of the p75NTR-specific ER-intrabody effect on regulating the p75NTR surface expression. Previous researches have suggested that ER-intrabody strategy led to a time-dependent downregulation of target protein expression. After transfection with specific ER-intrabodies, the surface expression of ErbB-2 (Curiel *et al.*, 2000) and VCAM-1 (Strebe *et al.*, 2009) had been progressively suppressed from 48 to 96 hr. Consistent with these results, the maximal efficiency of p75NTR knockdown by the ER-intrabody SH325-G7-KDEL appeared four days after transfection, and the effect persisted more than eight days post-transfection, although the p75NTR surface expression was increased slightly. In contrast to siRNA which normally maintains its effect on target knockdown for 3-5 days (Zou *et al.*, 2007), the long turnover kinetics of the ER-intrabody effect facilitates the investigation of the role of p75NTR in nerve system over a long period.

It is known that overexpression of heterologous proteins might saturate the cell's capacity for the protein folding, causing ER stress and resulting in the UPR initiation (Raden *et al.*, 2005). If the proper protein folding is not restored, the activation of UPR will signal an apoptotic response (Meusser *et al.*, 2005; Schroder, 2008). Moreover, the prolonged ER stress could lead to neurons death and arise the pathogenesis of neurodegenerative disorders (Malhotra and Kaufman, 2007). Hence, it is important to determine whether the UPR is activated during the knockdown process by the p75NTR-specific ER-intrabody. The ER-resident molecular chaperone GRP94 is one of the ER stress indicators since it has been demonstrated that the expression of GRP94 is upregulated by the UPR initiation (Bando *et al.*, 2003). GRP94 plays a pivotal role in the UPR by slowing down the protein folding process and marking unfolded proteins (Schroder, 2008). The expression levels of GRP94 were evaluated by immunoblotting in order to indicate the UPR in the PC12 cells expressing the ER-intrabody SH325-G7-KDEL. In comparison with DMSO treated PC12 cells, the expression levels of GRP94 were not increased in transfected PC12 cells during the time course knockdown experiment over eight days. However, tunicamycin, an UPR stimulus, considerably provoked the GRP94 expression in PC12 cells. Thus, the UPR was not activated by the ER-intrabody SH325-G7-KDEL transfection and expression. Furthermore, it implicates that the complexes

of ER-intrabodies and p75NTR may not accumulated within the ER, but be degraded via ER-associated degradation (ERAD) pathway (Meusser *et al.*, 2005).

Finally, the ER-intrabody SH325-G7-KDEL was applied to mouse hippocampal primary cultures prepared from mice (C57 Bl/6) embryonic day 18. Although p75NTR only abundantly expresses during development period, it is known to be present in hippocampal neurons of adult brains of mouse, rat, and human (Koh *et al.*, 1989; Kerwin *et al.*, 1993; Catts *et al.*, 2008). These findings imply that p75NTR has a critical role in modulating many aspects of hippocampal functions and behaviors. Since p75NTR is only present in about half of the pyramidal neurons of the CA1-CA4 subfields of hippocampus (Hu *et al.*, 2002), the endogenously p75NTR-positive and -negative neurons were indicated with *α*phOx-KDEL or fGFP transfection. Nonetheless, the p75NTR-positive neurons were also found in SH325-G7-KDEL transfected neurons, in which p75NTR surface expressions were supposed to be completely eliminated. It might arise from the inconstant expression of p75NTR in the p75NTR-expressing pyramidal neurons (Zagrebelsky *et al.*, 2005). The expression level of the ER-intrabody SH325-G7-KDEL may be efficient for some neurons but inefficient for the others to abolish the p75NTR surface expression completely.

In order to study the effect of the ER-intrabody in neurons, dendritic morphologies of transfected neurons were analyzed by fluorescence microscopy according to the intense labeling by EGFP-F. It is revealed that the dendritic complexity was significantly increased in the proximal region of the SH325-G7-KDEL transfected p75NTR-negative neurons, compared to the p75NTR-positive neurons (Fig. 4.16A, B). However, the endogenously p75NTR-negative neurons cannot be distinguished from the p75NTR-negative neurons due to the ER-intrabody effect in the SH325-G7-KDEL transfected neurons. Therefore, further analyses were performed in order to determine if the increase in the dendritic complexity was caused by the p75NTR surface knockdown. There were no significant changes in dendritic morphology observed between the endogenously p75NTR-positive and -negative neurons that were indicated with *α*phOx-KDEL or fGFP transfection (Fig. 4.16C, D). Taking into account that dendritic growth and branching are affected by many other intrinsic and extrinsic factors (McAllister *et al.*, 1997; Threadgill *et al.*, 1997; Hakeda-Suzuki *et al.*, 2002; Luo, 2002; Wang *et al.*, 2002; Gao and Bogert, 2003), it implies that the endogenously p75NTR-negative neurons may be negatively modulated in their dendritic complexities in some mechanisms other than p75NTR, and may behavior different from the endogenously p75NTR-positive neurons. Hence, the increase in the dendritic complexity is owing to the inhibition of p75NTR surface translocation by the ER-intrabody SH325-G7-KDEL. It likely indicates that p75NTR might be one of the factors that suppressed dendritic branching in mouse hippocampal neurons. This result was highly consistent with the previous finding that p75NTR negatively modulated dendrite complexity and spine density in hippocampal neurons (Zagrebelsky *et al.*, 2005).

In summary, three p75NTR-specific scFvs with nanomolar affinities were selected by phage display. The p75NTR-specific ER-intrabodies were generated by adding the C-terminal ER retention signal KDEL, and the construct SH325-G7-KDEL showed a remarkable effect on downregulating the p75NTR surface expression in PC12 and NSC19 cells. The effect of SH325-G7-KDEL maintained more than eight days on suppressing the surface translocation of p75NTR in PC12 cells, without obviously activating the UPR. It was also demonstrated that the intracellular expression level of ER-intrabody intensely influences the effect of ER-intrabody on the p75NTR surface knockdown. Finally, the p75NTR surface knockdown was determined in the mouse hippocampal primary cultures by SH325-G7-KDEL. It implicates that p75NTR may act essentially on modulating the dendritic morphology of hippocampal neurons, since the dendritic complexity was significantly increased when the p75NTR surface expression was reduced by the ER-intrabody.

6 Outlook

As shown in this study, the low intracellular expression level of ER-intrabody resulted in the inefficient target knockdown, while SH325-G7-KDEL with high expression efficiency could intensely downregulate the p75NTR surface expression in mammalian cells. However, the expression level of ER-intrabody cannot be unlimitedly enhanced because overexpressions of heterologous proteins may initiate the UPR in the cells and lead to cell apoptosis. Thus, increasing the binding affinity and avidity of ER-intrabody became necessary to improve the target surface knockdown. Many efforts have been investigated for antibody engineering to obtain higher affinity and avidity, increased half-life and stability. The affinities of antibody fragments could be augmented through targeted mutagenesis of CDRs or error prone PCR (Osbourn *et al.*, 1996; Colby *et al.*, 2004). In order to increase the avidity, bivalent antibody fragments can be produced by engineering an Fc domain to the C-terminal of scFv or constructing scFvs as diabodies (Holliger *et al.*, 1993; Demarest and Glaser, 2008). Furthermore, the *in vivo* stability of an antibody fragment will be dramatically improved as a result of the change of a scFv fragment into a Fab format (Quintero-Hernandez *et al.*, 2007).

In addition, multiple-target knockdown will be approached in the future from a single transcript according to the cap-independent translation mechanism directed by a mutant IRES (Li *et al.*, 2007). This IRES was genetically modified without obviously losing the translation efficiency in order that a new ER-intrabody could be inserted with one single cloning step.

The dendritic morphology of mouse hippocampal primary neurons was shown significantly modulated by the p75NTR-specific ER-intrabody SH325-G7-KDEL. However, the distinct apical and basal dendrites are often failed to be formed in the pyramidal neurons from dissociated cultures (Dotti *et al.*, 1988; Collin *et al.*, 1997). It seems that the dissociated primary culture is not the entirely analogue of hippocampus. Therefore, hippocampal organotypic slices need to be applied to investigate the substantial roles of p75NTR.

Although several approaches had been developed to silence the particular target expressions, ER-intrabody knockdown technology became one promising method to neutralize and study the functions of specific secretory proteins. The less immunogenic response might provide this technology to be more potential in the therapeutically applications, for example neurodegenerative diseases (Taylor *et al.*, 2002). In the past decades, more and more intrabodies had been generated to use in a wide range of applications, including HIV infection, tumor therapy, tissue transplantation, and treatments of neurological disorders (Kontermann, 2004; Miller and Messer, 2005; Boldicke, 2007). Moreover, proteome binder project has been started to generate recombinant antibodies against all human proteins, including variant forms and modifications. Thus ER-intrabodies

can be easily generated based on these present recombinant antibodies to investigate the functions of specific human surface proteins, including the uncharacterized, by inhibiting them from surface translocations.

7 References

Arevalo, J. C. and Wu, S. H. (2006). "Neurotrophin signaling: many exciting surprises!" Cell Mol Life Sci **63**(13): 1523-37.

Azzazy, H. M. and Highsmith, W. E., Jr. (2002). "Phage display technology: clinical applications and recent innovations." Clin Biochem **35**(6): 425-45.

Bando, Y., Katayama, T., Kasai, K., Taniguchi, M., Tamatani, M. and Tohyama, M. (2003). "GRP94 (94 kDa glucose-regulated protein) suppresses ischemic neuronal cell death against ischemia/reperfusion injury." Eur J Neurosci **18**(4): 829-40.

Barbas, C. F., 3rd, Kang, A. S., Lerner, R. A. and Benkovic, S. J. (1991). "Assembly of combinatorial antibody libraries on phage surfaces: the gene III site." Proc Natl Acad Sci U S A **88**(18): 7978-82.

Barker, P. A. (2004). "p75NTR is positively promiscuous: novel partners and new insights." Neuron **42**(4): 529-33.

Beattie, M. S., Harrington, A. W., Lee, R., Kim, J. Y., Boyce, S. L., Longo, F. M., Bresnahan, J. C., Hempstead, B. L. and Yoon, S. O. (2002). "ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury." Neuron **36**(3): 375-86.

Bender, L. M. and Thorburn, A. (2005). "Death Domain Interactions in Death Receptor Signaling." Journal of biological sciences **5**(1): 33-37.

Better, M., Chang, C. P., Robinson, R. R. and Horwitz, A. H. (1988). "Escherichia coli secretion of an active chimeric antibody fragment." Science **240**(4855): 1041-3.

Bibel, M., Hoppe, E. and Barde, Y. A. (1999). "Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR." EMBO J **18**(3): 616-22.

Boder, E. T. and Wittrup, K. D. (1997). "Yeast surface display for screening combinatorial polypeptide libraries." Nat Biotechnol **15**(6): 553-7.

Boldicke, T. (2007). "Blocking translocation of cell surface molecules from the ER to the cell surface by intracellular antibodies targeted to the ER." J Cell Mol Med **11**(1): 54-70.

Breitling, F., Dubel, S., Seehaus, T., Klewinghaus, I. and Little, M. (1991). "A surface expression vector for antibody screening." Gene **104**(2): 147-53.

Carson, M., Johnson, D. H., McDonald, H., Brouillette, C. and Delucas, L. J. (2007). "His-tag impact on structure." Acta Crystallogr D Biol Crystallogr **63**(Pt 3): 295-301.

Carter, B. D., Kaltschmidt, C., Kaltschmidt, B., Offenhauser, N., Bohm-Matthaei, R., Baeuerle, P. A. and Barde, Y. A. (1996). "Selective activation of NF-kappa B by nerve growth factor through the neurotrophin receptor p75." Science **272**(5261): 542-5.

Cashman, N. R., Durham, H. D., Blusztajn, J. K., Oda, K., Tabira, T., Shaw, I. T., Dahrouge, S. and Antel, J. P. (1992). "Neuroblastoma x spinal cord (NSC) hybrid cell lines resemble developing motor neurons." Dev Dyn **194**(3): 209-21.

Catts, V. S., Al-Menhali, N., Burne, T. H., Colditz, M. J. and Coulson, E. J. (2008). "The p75 neurotrophin receptor regulates hippocampal neurogenesis and related behaviours." Eur J Neurosci **28**(5): 883-92.

Chao, M. V. (2003). "Neurotrophins and their receptors: a convergence point for many signalling pathways." Nat Rev Neurosci **4**(4): 299-309.

Clackson, T., Hoogenboom, H. R., Griffiths, A. D. and Winter, G. (1991). "Making antibody fragments using phage display libraries." Nature **352**(6336): 624-8.

Colby, D. W., Kellogg, B. A., Graff, C. P., Yeung, Y. A., Swers, J. S. and Wittrup, K. D. (2004). "Engineering antibody affinity by yeast surface display." Methods Enzymol **388**: 348-58.

Collin, C., Miyaguchi, K. and Segal, M. (1997). "Dendritic spine density and LTP induction in cultured hippocampal slices." J Neurophysiol **77**(3): 1614-23.

Coulson, E. J., Reid, K., Baca, M., Shipham, K. A., Hulett, S. M., Kilpatrick, T. J. and Bartlett, P. F. (2000). "Chopper, a new death domain of the p75 neurotrophin receptor that mediates rapid neuronal cell death." J Biol Chem **275**(39): 30537-45.

Couzin, J. (2004). "Molecular biology. RNAi shows cracks in its armor." Science **306**(5699): 1124-5.

Curiel, D. T., Stackhouse, M. and Buchsbaum, D. J. (2000). "Enhancement of tumor cell radiosensitivity using single chain intracellular antibodies - US Patent 6074640 Available at: http://www.patentstorm.us/patents/6074640/fulltext.html."

de Haard, H. J., van Neer, N., Reurs, A., Hufton, S. E., Roovers, R. C., Henderikx, P., de Bruine, A. P., Arends, J. W. and Hoogenboom, H. R. (1999). "A large non-immunized human Fab fragment phage library that permits rapid isolation and kinetic analysis of high affinity antibodies." J Biol Chem **274**(26): 18218-30.

Dechant, G. and Barde, Y. A. (1997). "Signalling through the neurotrophin receptor p75NTR." Curr Opin Neurobiol **7**(3): 413-8.

Dechant, G. and Barde, Y. A. (2002). "The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system." Nat Neurosci **5**(11): 1131-6.

Delves, P., Martin, S., Burton, D., and Roitt, I. (2006). "Roitt's Essential Immunology, 11th Edition." Wiley-Blackwell.

Demarest, S. J. and Glaser, S. M. (2008). "Antibody therapeutics, antibody engineering, and the merits of protein stability." Curr Opin Drug Discov Devel **11**(5): 675-87.

Deshane, J., Siegal, G. P., Wang, M., Wright, M., Bucy, R. P., Alvarez, R. D. and Curiel, D. T. (1997). "Transductional efficacy and safety of an intraperitoneally delivered adenovirus encoding an anti-erbB-2 intracellular single-chain antibody for ovarian cancer gene therapy." Gynecol Oncol **64**(3): 378-85.

Dotti, C. G., Sullivan, C. A. and Banker, G. A. (1988). "The establishment of polarity by hippocampal neurons in culture." J Neurosci **8**(4): 1454-68.

Dübel, S. (2007). "Handbook of Therapeutic Antibodies, 1st Editon " Wiley-VCH.

Dübel, S., Breitling, F., Klewinghaus, I. and Little, M. (1992). "Regulated secretion and purification of recombinant antibodies in E. coli." Cell Biophys **21**(1-3): 69-79.

Epa, W. R., Markovska, K. and Barrett, G. L. (2004). "The p75 neurotrophin receptor enhances TrkA signalling by binding to Shc and augmenting its phosphorylation." J Neurochem **89**(2): 344-53.

Famulok, M., Mayer, G. and Blind, M. (2000). "Nucleic acid aptamers-from selection in vitro to applications in vivo." Acc Chem Res **33**(9): 591-9.

Fish, R. J. and Kruithof, E. K. (2004). "Short-term cytotoxic effects and long-term instability of RNAi delivered using lentiviral vectors." BMC Mol Biol **5**: 9.

Fournier, A. E., GrandPre, T. and Strittmatter, S. M. (2001). "Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration." Nature **409**(6818): 341-6.

Fuchs, P., Breitling, F., Dubel, S., Seehaus, T. and Little, M. (1991). "Targeting recombinant antibodies to the surface of Escherichia coli: fusion to a peptidoglycan associated lipoprotein." Biotechnology (N Y) **9**(12): 1369-72.

Gao, F. B. and Bogert, B. A. (2003). "Genetic control of dendritic morphogenesis in Drosophila." Trends Neurosci **26**(5): 262-8.

Gehler, S., Gallo, G., Veien, E. and Letourneau, P. C. (2004). "p75 neurotrophin receptor signaling regulates growth cone filopodial dynamics through modulating RhoA activity." J Neurosci **24**(18): 4363-72.

Gentry, J. J., Rutkoski, N. J., Burke, T. L. and Carter, B. D. (2004b). "A functional interaction between the p75 neurotrophin receptor interacting factors, TRAF6 and NRIF." J Biol Chem **279**(16): 16646-56.

Gong, Y., Cao, P., Yu, H. J. and Jiang, T. (2008). "Crystal structure of the neurotrophin-3 and p75NTR symmetrical complex." Nature **454**(7205): 789-93.

Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R. E., Jones, P. T., Low, N. M., Allison, T. J. and et al. (1994). "Isolation

of high affinity human antibodies directly from large synthetic repertoires." EMBO J **13**(14): 3245-60.

Hakeda-Suzuki, S., Ng, J., Tzu, J., Dietzl, G., Sun, Y., Harms, M., Nardine, T., Luo, L. and Dickson, B. J. (2002). "Rac function and regulation during Drosophila development." Nature **416**(6879): 438-42.

Hanes, J. and Pluckthun, A. (1997). "In vitro selection and evolution of functional proteins by using ribosome display." Proc Natl Acad Sci U S A **94**(10): 4937-42.

Harrington, A. W., Leiner, B., Blechschmitt, C., Arevalo, J. C., Lee, R., Morl, K., Meyer, M., Hempstead, B. L., Yoon, S. O. and Giehl, K. M. (2004). "Secreted proNGF is a pathophysiological death-inducing ligand after adult CNS injury." Proc Natl Acad Sci U S A **101**(16): 6226-30.

Hashimoto, Y., Kaneko, Y., Tsukamoto, E., Frankowski, H., Kouyama, K., Kita, Y., Niikura, T., Aiso, S., Bredesen, D. E., Matsuoka, M. and Nishimoto, I. (2004). "Molecular characterization of neurohybrid cell death induced by Alzheimer's amyloid-beta peptides via p75NTR/PLAIDD." J Neurochem **90**(3): 549-58.

Hawlisch, H., Muller, M., Frank, R., Bautsch, W., Klos, A. and Kohl, J. (2001). "Site-specific anti-C3a receptor single-chain antibodies selected by differential panning on cellulose sheets." Anal Biochem **293**(1): 142-5.

Hayashi, N., Welschof, M., Zewe, M., Braunagel, M., Dubel, S., Breitling, F. and Little, M. (1994). "Simultaneous mutagenesis of antibody CDR regions by overlap extension and PCR." Biotechniques **17**(2): 310, 312, 314-5.

He, X. L. and Garcia, K. C. (2004). "Structure of nerve growth factor complexed with the shared neurotrophin receptor p75." Science **304**(5672): 870-5.

He, Z. and Koprivica, V. (2004). "The Nogo signaling pathway for regeneration block." Annu Rev Neurosci **27**: 341-68.

Hempstead, B. L. (2002). "The many faces of p75NTR." Curr Opin Neurobiol **12**(3): 260-7.

Holliger, P., Prospero, T. and Winter, G. (1993). ""Diabodies": small bivalent and bispecific antibody fragments." Proc Natl Acad Sci U S A **90**(14): 6444-8.

Hu, X. Y., Zhang, H. Y., Qin, S., Xu, H., Swaab, D. F. and Zhou, J. N. (2002). "Increased p75(NTR) expression in hippocampal neurons containing hyperphosphorylated tau in Alzheimer patients." Exp Neurol **178**(1): 104-11.

Hust, M. and Dübel, S. (2005). "Phage display vectors for the in vitro generation of human antibody fragments." Methods Mol Biol **295**: 71-96.

Hust, M., Dubel, S. and Schirrmann, T. (2007c). "Selection of recombinant antibodies from antibody gene libraries." Methods Mol Biol **408**: 243-55.

Hust, M., Jostock, T., Menzel, C., Voedisch, B., Mohr, A., Brenneis, M., Kirsch, M. I., Meier, D. and Dubel, S. (2007a). "Single chain Fab (scFab) fragment." BMC Biotechnol **7**: 14.

Hust, M., Maiss, E., Jacobsen, H. J. and Reinard, T. (2002). "The production of a genus-specific recombinant antibody (scFv) using a recombinant potyvirus protease." J Virol Methods **106**(2): 225-33.

Hust, M., Toleikis, L. and Dübel, S. (2007b). "Antibody phage display. In the handbook of therapeutic antibodies. Edited by Dübel, S." Weinheim: Willey-VCH: 45 - 68.

Ip, N. Y., Stitt, T. N., Tapley, P., Klein, R., Glass, D. J., Fandl, J., Greene, L. A., Barbacid, M. and Yancopoulos, G. D. (1993). "Similarities and differences in the way neurotrophins interact with the Trk receptors in neuronal and nonneuronal cells." Neuron **10**(2): 137-49.

Jacob, F. and Monod, J. (1961). "Genetic regulatory mechanisms in the synthesis of proteins." J Mol Biol **3**: 318-56.

Jefferis, R., Lund, J. and Pound, J. D. (1998). "IgG-Fc-mediated effector functions: molecular definition of interaction sites for effector ligands and the role of glycosylation." Immunol Rev **163**: 59-76.

Johnson, D., Lanahan, A., Buck, C. R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M. and Chao, M. (1986). "Expression and structure of the human NGF receptor." Cell **47**(4): 545-54.

Jung, K. M., Tan, S., Landman, N., Petrova, K., Murray, S., Lewis, R., Kim, P. K., Kim, D. S., Ryu, S. H., Chao, M. V. and Kim, T. W. (2003). "Regulated intramembrane proteolysis of the p75 neurotrophin receptor modulates its association with the TrkA receptor." J Biol Chem **278**(43): 42161-9.

Kabat, E. A., Wu, T. T. and Bilofsky, H. (1979). "Evidence supporting somatic assembly of the DNA segments (minigenes), coding for the framework, and complementarity-determining segments of immunoglobulin variable regions." J Exp Med **149**(6): 1299-313.

Kalejta, R. F., Shenk, T. and Beavis, A. J. (1997). "Use of a membrane-localized green fluorescent protein allows simultaneous identification of transfected cells and cell cycle analysis by flow cytometry." Cytometry **29**(4): 286-91.

Kanning, K. C., Hudson, M., Amieux, P. S., Wiley, J. C., Bothwell, M. and Schecterson, L. C. (2003). "Proteolytic processing of the p75 neurotrophin receptor and two homologs generates C-terminal fragments with signaling capability." J Neurosci **23**(13): 5425-36.

Kerwin, J. M., Morris, C. M., Johnson, M., Perry, R. H. and Perry, E. K. (1993). "Hippocampal p75 nerve growth factor receptor immunoreactivity in development, normal aging and senescence." Acta Anat (Basel) **147**(4): 216-22.

Kirsch, M. I., Hulseweh, B., Nacke, C., Rulker, T., Schirrmann, T., Marschall, H. J., Hust, M. and Dubel, S. (2008). "Development of human antibody fragments using antibody phage display for the detection and diagnosis of Venezuelan equine encephalitis virus (VEEV)." BMC Biotechnol **8**: 66.

Koh, S., Oyler, G. A. and Higgins, G. A. (1989). "Localization of nerve growth factor receptor messenger RNA and protein in the adult rat brain." Exp Neurol **106**(3): 209-21.

Köhler, G. and Milstein, C. (1975). "Continuous cultures of fused cells secreting antibody of predefined specificity." Nature **256**(5517): 495-7.

Kontermann, R. and Dübel, S. (2001). "Antibody engineering." Springer Lab Manual.

Kontermann, R. E. (2004). "Intrabodies as therapeutic agents." Methods **34**(2): 163-70.

Kugler, J., Nieswandt, S., Gerlach, G. F., Meens, J., Schirrmann, T. and Hust, M. (2008). "Identification of immunogenic polypeptides from a Mycoplasma hyopneumoniae genome library by phage display." Appl Microbiol Biotechnol **80**(3): 447-58.

Kuruvilla, R., Zweifel, L. S., Glebova, N. O., Lonze, B. E., Valdez, G., Ye, H. and Ginty, D. D. (2004). "A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling." Cell **118**(2): 243-55.

Lad, S. P., Peterson, D. A., Bradshaw, R. A. and Neet, K. E. (2003). "Individual and combined effects of TrkA and p75NTR nerve growth factor receptors. A role for the high affinity receptor site." J Biol Chem **278**(27): 24808-17.

Lee, R., Kermani, P., Teng, K. K. and Hempstead, B. L. (2001). "Regulation of cell survival by secreted proneurotrophins." Science **294**(5548): 1945-8.

Lewis, M. J. and Pelham, H. R. (1992a). "Ligand-induced redistribution of a human KDEL receptor from the Golgi complex to the endoplasmic reticulum." Cell **68**(2): 353-64.

Lewis, M. J. and Pelham, H. R. (1992b). "Sequence of a second human KDEL receptor." J Mol Biol **226**(4): 913-6.

Li, J., Zhang, C., Jostock, T. and Dubel, S. (2007). "Analysis of IgG heavy chain to light chain ratio with mutant Encephalomyocarditis virus internal ribosome entry site." Protein Eng Des Sel **20**(10): 491-6.

Liu, Y., Majumder, S., McCall, W., Sartor, C. I., Mohler, J. L., Gregory, C. W., Earp, H. S. and Whang, Y. E. (2005). "Inhibition of HER-2/neu kinase impairs androgen receptor recruitment to the androgen responsive enhancer." Cancer Res **65**(8): 3404-9.

Lu, B., Pang, P. T. and Woo, N. H. (2005). "The yin and yang of neurotrophin action." Nat Rev Neurosci **6**(8): 603-14.

Lund, J., Takahashi, N., Pound, J. D., Goodall, M. and Jefferis, R. (1996). "Multiple interactions of IgG with its core oligosaccharide can modulate recognition by

complement and human Fc gamma receptor I and influence the synthesis of its oligosaccharide chains." J Immunol **157**(11): 4963-9.
Luo, L. (2002). "Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity." Annu Rev Cell Dev Biol **18**: 601-35.
Mahoney, W. C. and Duksin, D. (1979). "Biological activities of the two major components of tunicamycin." J Biol Chem **254**(14): 6572-6.
Majdan, M., Lachance, C., Gloster, A., Aloyz, R., Zeindler, C., Bamji, S., Bhakar, A., Belliveau, D., Fawcett, J., Miller, F. D. and Barker, P. A. (1997). "Transgenic mice expressing the intracellular domain of the p75 neurotrophin receptor undergo neuronal apoptosis." J Neurosci **17**(18): 6988-98.
Malhotra, J. D. and Kaufman, R. J. (2007). "The endoplasmic reticulum and the unfolded protein response." Semin Cell Dev Biol **18**(6): 716-31.
McAllister, A. K., Katz, L. C. and Lo, D. C. (1997). "Opposing roles for endogenous BDNF and NT-3 in regulating cortical dendritic growth." Neuron **18**(5): 767-78.
McCafferty, J., Griffiths, A. D., Winter, G. and Chiswell, D. J. (1990). "Phage antibodies: filamentous phage displaying antibody variable domains." Nature **348**(6301): 552-4.
Mead, D. A. and Kemper, B. (1988). "Chimeric single-stranded DNA phage-plasmid cloning vectors." Biotechnology **10**: 85-102.
Meusser, B., Hirsch, C., Jarosch, E. and Sommer, T. (2005). "ERAD: the long road to destruction." Nat Cell Biol **7**(8): 766-72.
Mi, S., Lee, X., Shao, Z., Thill, G., Ji, B., Relton, J., Levesque, M., Allaire, N., Perrin, S., Sands, B., Crowell, T., Cate, R. L., McCoy, J. M. and Pepinsky, R. B. (2004). "LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex." Nat Neurosci **7**(3): 221-8.
Miller, T. W. and Messer, A. (2005). "Intrabody applications in neurological disorders: progress and future prospects." Mol Ther **12**(3): 394-401.
Mocellin, S. and Provenzano, M. (2004). "RNA interference: learning gene knock-down from cell physiology." J Transl Med **2**(1): 39.
Moghaddam, A., Borgen, T., Stacy, J., Kausmally, L., Simonsen, B., Marvik, O. J., Brekke, O. H. and Braunagel, M. (2003). "Identification of scFv antibody fragments that specifically recognise the heroin metabolite 6-monoacetylmorphine but not morphine." J Immunol Methods **280**(1-2): 139-55.
Mukai, J., Hachiya, T., Shoji-Hoshino, S., Kimura, M. T., Nadano, D., Suvanto, P., Hanaoka, T., Li, Y., Irie, S., Greene, L. A. and Sato, T. A. (2000). "NADE, a p75NTR-associated cell death executor, is involved in signal transduction mediated by the common neurotrophin receptor p75NTR." J Biol Chem **275**(23): 17566-70.
Munro, S. and Pelham, H. R. (1987). "A C-terminal signal prevents secretion of luminal ER proteins." Cell **48**(5): 899-907.
Nykjaer, A., Lee, R., Teng, K. K., Jansen, P., Madsen, P., Nielsen, M. S., Jacobsen, C., Kliemannel, M., Schwarz, E., Willnow, T. E., Hempstead, B. L. and Petersen, C. M. (2004). "Sortilin is essential for proNGF-induced neuronal cell death." Nature **427**(6977): 843-8.
Nykjaer, A., Willnow, T. E. and Petersen, C. M. (2005). "p75NTR--live or let die." Curr Opin Neurobiol **15**(1): 49-57.
Olden, K., Pratt, R. M., Jaworski, C. and Yamada, K. M. (1979). "Evidence for role of glycoprotein carbohydrates in membrane transport: specific inhibition by tunicamycin." Proc Natl Acad Sci U S A **76**(2): 791-5.
Osbourn, J. K., Field, A., Wilton, J., Derbyshire, E., Earnshaw, J. C., Jones, P. T., Allen, D. and McCafferty, J. (1996). "Generation of a panel of related human scFv antibodies with high affinities for human CEA." Immunotechnology **2**(3): 181-96.
Paganetti, P., Calanca, V., Galli, C., Stefani, M. and Molinari, M. (2005). "beta-site specific intrabodies to decrease and prevent generation of Alzheimer's Abeta peptide." J Cell Biol **168**(6): 863-8.

Parmley, S. F. and Smith, G. P. (1988). "Antibody-selectable filamentous fd phage vectors: affinity purification of target genes." Gene **73**(2): 305-18.

Paul, C. E., Vereker, E., Dickson, K. M. and Barker, P. A. (2004). "A pro-apoptotic fragment of the p75 neurotrophin receptor is expressed in p75NTRExonIV null mice." J Neurosci **24**(8): 1917-23.

Pini, A., Viti, F., Santucci, A., Carnemolla, B., Zardi, L., Neri, P. and Neri, D. (1998). "Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel." J Biol Chem **273**(34): 21769-76.

Pluckthun, A. (1990). "Antibodies from Escherichia coli." Nature **347**(6292): 497-8.

Quintero-Hernandez, V., Juarez-Gonzalez, V. R., Ortiz-Leon, M., Sanchez, R., Possani, L. D. and Becerril, B. (2007). "The change of the scFv into the Fab format improves the stability and in vivo toxin neutralization capacity of recombinant antibodies." Mol Immunol **44**(6): 1307-15.

Rabizadeh, S., Oh, J., Zhong, L. T., Yang, J., Bitler, C. M., Butcher, L. L. and Bredesen, D. E. (1993). "Induction of apoptosis by the low-affinity NGF receptor." Science **261**(5119): 345-8.

Raden, D., Hildebrandt, S., Xu, P., Bell, E., Doyle, F. J., 3rd and Robinson, A. S. (2005). "Analysis of cellular response to protein overexpression." Syst Biol (Stevenage) **152**(4): 285-9.

Roberts, R. W. and Szostak, J. W. (1997). "RNA-peptide fusions for the in vitro selection of peptides and proteins." Proc Natl Acad Sci U S A **94**(23): 12297-302.

Rondot, S., Koch, J., Breitling, F. and Dubel, S. (2001). "A helper phage to improve single-chain antibody presentation in phage display." Nat Biotechnol **19**(1): 75-8.

Rosch, H., Schweigreiter, R., Bonhoeffer, T., Barde, Y. A. and Korte, M. (2005). "The neurotrophin receptor p75NTR modulates long-term depression and regulates the expression of AMPA receptor subunits in the hippocampus." Proc Natl Acad Sci U S A **102**(20): 7362-7.

Russel, M., Linderoth, N. A. and Sali, A. (1997). "Filamentous phage assembly: variation on a protein export theme." Gene **192**(1): 23-32.

Salama-Cohen, P., Arevalo, M. A., Meier, J., Grantyn, R. and Rodriguez-Tebar, A. (2005). "NGF controls dendrite development in hippocampal neurons by binding to p75NTR and modulating the cellular targets of Notch." Mol Biol Cell **16**(1): 339-47.

Salehi, A. H., Roux, P. P., Kubu, C. J., Zeindler, C., Bhakar, A., Tannis, L. L., Verdi, J. M. and Barker, P. A. (2000). "NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis." Neuron **27**(2): 279-88.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual, 2nd ed." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanna, P. P., Williamson, R. A., De Logu, A., Bloom, F. E. and Burton, D. R. (1995). "Directed selection of recombinant human monoclonal antibodies to herpes simplex virus glycoproteins from phage display libraries." Proc Natl Acad Sci U S A **92**(14): 6439-43.

Santee, S. M. and Owen-Schaub, L. B. (1996). "Human tumor necrosis factor receptor p75/80 (CD120b) gene structure and promoter characterization." J Biol Chem **271**(35): 21151-9.

Sasaki, T. and Takai, Y. (1998). "The Rho small G protein family-Rho GDI system as a temporal and spatial determinant for cytoskeletal control." Biochem Biophys Res Commun **245**(3): 641-5.

Schirrmann, T., Al-Halabi, L., Dubel, S. and Hust, M. (2008). "Production systems for recombinant antibodies." Front Biosci **13**: 4576-94.

Schmiedl, A., Breitling, F. and Dubel, S. (2000). "Expression of a bispecific dsFv-dsFv' antibody fragment in Escherichia coli." Protein Eng **13**(10): 725-34.

Schor, N. F. (2005). "The p75 neurotrophin receptor in human development and disease." Prog Neurobiol **77**(3): 201-14.

Schouten, A., Roosien, J., van Engelen, F. A., de Jong, G. A., Borst-Vrenssen, A. W., Zilverentant, J. F., Bosch, D., Stiekema, W. J., Gommers, F. J., Schots, A. and Bakker, J. (1996). "The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco." Plant Mol Biol **30**(4): 781-93.

Schroder, M. (2008). "Endoplasmic reticulum stress responses." Cell Mol Life Sci **65**(6): 862-94.

Schütte, M., Thullier, P., Pelat, T., Wezler, X., Rosenstock, P., Hinz, D., Kirsch, M. I., Hasenberg, M., Frank, R., Schirrmann, T., Gunzer, M., Hust, M. and Dubel, S. (2009). "Identification of a putative Crf splice variant and generation of recombinant antibodies for the specific detection of Aspergillus fumigatus." PLoS One **4**(8): e6625.

Sholl, D. A. (1953). "Dendritic organization in the neurons of the visual and motor cortices of the cat." J Anat **87**(4): 387-406.

Skerra, A. and Pluckthun, A. (1988). "Assembly of a functional immunoglobulin Fv fragment in Escherichia coli." Science **240**(4855): 1038-41.

Sledz, C. A., Holko, M., de Veer, M. J., Silverman, R. H. and Williams, B. R. (2003). "Activation of the interferon system by short-interfering RNAs." Nat Cell Biol **5**(9): 834-9.

Smith, G. P. (1985). "Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface." Science **228**(4705): 1315-7.

Sole, C., Dolcet, X., Segura, M. F., Gutierrez, H., Diaz-Meco, M. T., Gozzelino, R., Sanchis, D., Bayascas, J. R., Gallego, C., Moscat, J., Davies, A. M. and Comella, J. X. (2004). "The death receptor antagonist FAIM promotes neurite outgrowth by a mechanism that depends on ERK and NF-kapp B signaling." J Cell Biol **167**(3): 479-92.

Soltes, G., Hust, M., Ng, K. K., Bansal, A., Field, J., Stewart, D. I., Dubel, S., Cha, S. and Wiersma, E. J. (2007). "On the influence of vector design on antibody phage display." J Biotechnol **127**(4): 626-37.

Strebe, N., Guse, A., Schungel, M., Schirrmann, T., Hafner, M., Jostock, T., Hust, M., Muller, W. and Dubel, S. (2009). "Functional knockdown of VCAM-1 at the posttranslational level with ER retained antibodies." J Immunol Methods **341**(1-2): 30-40.

Taylor, J. P., Hardy, J. and Fischbeck, K. H. (2002). "Toxic proteins in neurodegenerative disease." Science **296**(5575): 1991-5.

Teng, H. K., Teng, K. K., Lee, R., Wright, S., Tevar, S., Almeida, R. D., Kermani, P., Torkin, R., Chen, Z. Y., Lee, F. S., Kraemer, R. T., Nykjaer, A. and Hempstead, B. L. (2005). "ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin." J Neurosci **25**(22): 5455-63.

Thie, H., Meyer, T., Schirrmann, T., Hust, M. and Dubel, S. (2008). "Phage display derived therapeutic antibodies." Curr Pharm Biotechnol **9**(6): 439-46.

Threadgill, R., Bobb, K. and Ghosh, A. (1997). "Regulation of dendritic growth and remodeling by Rho, Rac, and Cdc42." Neuron **19**(3): 625-34.

Vetrugno, V., Cardinale, A., Filesi, I., Mattei, S., Sy, M. S., Pocchiari, M. and Biocca, S. (2005). "KDEL-tagged anti-prion intrabodies impair PrP lysosomal degradation and inhibit scrapie infectivity." Biochem Biophys Res Commun **338**(4): 1791-7.

Vieira, J. and Messing, J. (1987). "Production of single-stranded plasmid DNA." Methods Enzymol **153**: 3-11.

von Schack, D., Casademunt, E., Schweigreiter, R., Meyer, M., Bibel, M. and Dechant, G. (2001). "Complete ablation of the neurotrophin receptor p75NTR causes defects both in the nervous and the vascular system." Nat Neurosci **4**(10): 977-8.

Wang, K. C., Kim, J. A., Sivasankaran, R., Segal, R. and He, Z. (2002). "P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp." Nature **420**(6911): 74-8.

Wehrman, T., He, X., Raab, B., Dukipatti, A., Blau, H. and Garcia, K. C. (2007). "Structural and mechanistic insights into nerve growth factor interactions with the TrkA and p75 receptors." Neuron **53**(1): 25-38.

Wilson, D. W., Lewis, M. J. and Pelham, H. R. (1993). "pH-dependent binding of KDEL to its receptor in vitro." J Biol Chem **268**(10): 7465-8.

Wong, S. T., Henley, J. R., Kanning, K. C., Huang, K. H., Bothwell, M. and Poo, M. M. (2002). "A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein." Nat Neurosci **5**(12): 1302-8.

Woo, N. H., Teng, H. K., Siao, C. J., Chiaruttini, C., Pang, P. T., Milner, T. A., Hempstead, B. L. and Lu, B. (2005). "Activation of p75NTR by proBDNF facilitates hippocampal long-term depression." Nat Neurosci **8**(8): 1069-77.

Woolf, C. J. (2003). "No Nogo: now where to go?" Neuron **38**(2): 153-6.

Yamashita, T., Fujitani, M., Hata, K., Mimura, F. and Yamagishi, S. (2005). "Diverse functions of the p75 neurotrophin receptor." Anat Sci Int **80**(1): 37-41.

Yamashita, T. and Tohyama, M. (2003). "The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI." Nat Neurosci **6**(5): 461-7.

Yan, A. C. and Levy, M. (2009). "Aptamers and aptamer targeted delivery." RNA Biol **6**(3): 316-20.

Yeiser, E. C., Rutkoski, N. J., Naito, A., Inoue, J. and Carter, B. D. (2004). "Neurotrophin signaling through the p75 receptor is deficient in traf6-/- mice." J Neurosci **24**(46): 10521-9.

Yoneda, Y., Semba, T., Kaneda, Y., Noble, R. L., Matsuoka, Y., Kurihara, T., Okada, Y. and Imamoto, N. (1992). "A long synthetic peptide containing a nuclear localization signal and its flanking sequences of SV40 T-antigen directs the transport of IgM into the nucleus efficiently." Exp Cell Res **201**(2): 313-20.

Yoon, S. O., Casaccia-Bonnefil, P., Carter, B. and Chao, M. V. (1998). "Competitive signaling between TrkA and p75 nerve growth factor receptors determines cell survival." J Neurosci **18**(9): 3273-81.

Zaccaro, M. C., Ivanisevic, L., Perez, P., Meakin, S. O. and Saragovi, H. U. (2001). "p75 Co-receptors regulate ligand-dependent and ligand-independent Trk receptor activation, in part by altering Trk docking subdomains." J Biol Chem **276**(33): 31023-9.

Zagrebelsky, M., Holz, A., Dechant, G., Barde, Y. A., Bonhoeffer, T. and Korte, M. (2005). "The p75 neurotrophin receptor negatively modulates dendrite complexity and spine density in hippocampal neurons." J Neurosci **25**(43): 9989-99.

Zhang, Y., Hong, Y., Bounhar, Y., Blacker, M., Roucou, X., Tounekti, O., Vereker, E., Bowers, W. J., Federoff, H. J., Goodyer, C. G. and LeBlanc, A. (2003). "p75 neurotrophin receptor protects primary cultures of human neurons against extracellular amyloid beta peptide cytotoxicity." J Neurosci **23**(19): 7385-94.

Zou, G. M., Thompson, M. A. and Yoder, M. C. (2007). "RNAi knockdown of transcription factor Pu.1 in the differentiation of mouse embryonic stem cells." Methods Mol Biol **407**: 127-36.